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EXPERIMENTAL CELL RESEARCH

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SYNCHRONOUS DIVISIONS IN *TETRAHYMENA PYRIFORMIS* AS STUDIED IN AN INORGANIC MEDIUM.

THE EFFECT OF 2,4-DINITROPHENOL[1]

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Received February 23, 1957

In *Tetrahymena pyriformis* grown on proteose-peptone [11, 15] and on synthetic media [12] cell divisions can be synchronized by applying a controlled temperature cycle. The synchronized cells are fairly large and the divisions occur faster than in the untreated log-phase cells. During synchronous division, growth is retarded, but it later returns to the normal rate for log-phase cells.

This paper will show that it is possible to eliminate growth altogether by transferring the heat-treated cells to an inorganic medium in which the synchronized division steps are preserved. We have attempted further characterization of the metabolism of these large synchronized cells by measuring respiration rates and by studying the influence of metabolic inhibitors. In this paper the effect of 2,4-dinitrophenol (DNP) is reported.

METHODS

The organism and the conditions under which it was grown and synchronized are described in reference [12]. Respiration was measured conventionally in the Warburg apparatus. The media used were 2 per cent proteose-peptone [12] in which the cells had been grown, or an inorganic medium: 2.75 g NaCl, 0.25 g $MgSO_4$, 7 H_2O made up to one liter with 5×10^{-3} *M* phosphate buffer. In different experiments pH was varied between 6.0 and 7.3. The untreated cultures were harvested in the logarithmic growth phase at a population density of 50,000–100,000 cells/ml. The synchronizing heat treatment was supplied at a population density of 20,000–50,000, and when harvested the synchronized population had this density. When studied in the proteose-peptone medium 3 ml of the culture was pipetted into the Warburg vessel (15–20 ml). When studied in the inorganic medium the cells were washed three times by hand centrifugation and concentrated to 100,000–200,000 log-phase

[1] Supported by grants from the Eli Lilly Foundation and from the Carlsberg Foundation.

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cells or to 50,000–75,000 synchronized cells per ml. Three milliliters of this suspension were introduced into the Warburg vessel. The side bulb was charged with 0.5 ml containing the calculated amount of DNP and the center well with 0.2 ml 10 per cent NaOH. Multiplication of cells in the Warburg vessels was followed by making counts before and after the respiration experiments. Also, the influence of DNP on cell multiplication was followed by counts and by observation of the times for the divi-

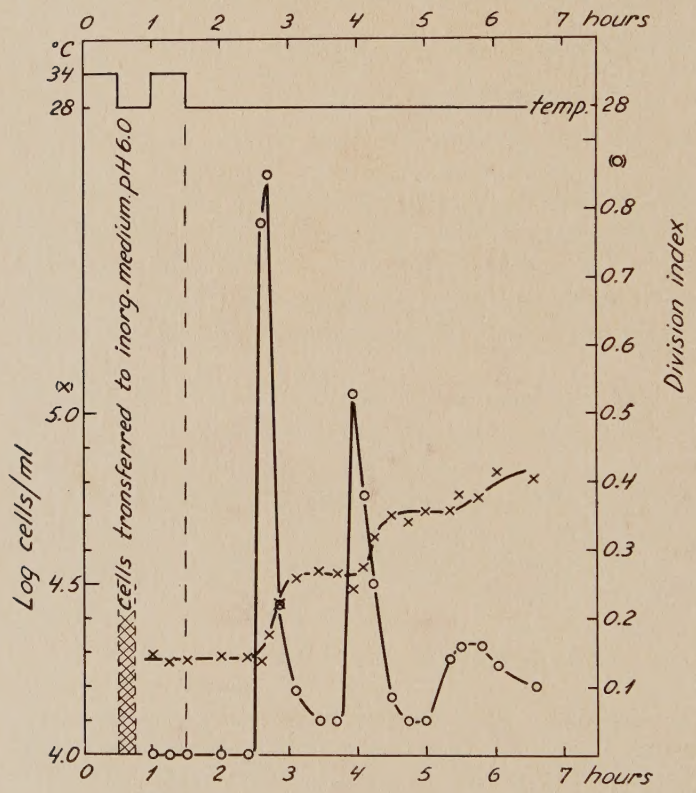

Fig. 1.—*Tetrahymena* synchronized with seven heat shocks in proteose-peptone, and one heat shock in inorganic medium.

sion maxima in 10–15 ml cultures in 50 ml Erlenmeyer flasks, which were kept shaking in the thermostat. For counting we used the Sedgewick-Rafter counting chamber [12].

RESULTS

Synchronized divisions in inorganic medium.—Cells grown on proteose-peptone at 28° were synchronized by exposure to 8 periods of elevated (34.0°) temperature. In the last period at 28°, before receiving the last temperature shock, the cells were transferred through three washings to the inor-

TABLE I

	μl O_2/10^6 cells/min	Number of experiments	mg dry weight per 10^6 cells
1. Culture in log phase proteose-peptone	4.8 ± 0.25^a	24	2.6
2. Culture in log phase inorganic medium	1.4 ± 0.04^a	39	—
3. Synchronized culture proteose-peptone	15.2 ± 0.12^a	6	7.5
4. Synchronized culture inorganic medium	4.6 ± 0.09^a	66	—

[a] Calculated by means of the formula $\pm\sqrt{\Sigma d^2/n(n-1)}$. Ratio: 2/1 = 0.29, and ratio 4/3 = 0.30.

ganic medium. At the same time they were concentrated by a factor of 2 to 3 for use in the Warburg apparatus.

After final transfer to 28° the heat-treated, washed and concentrated cell suspension exhibited synchronous division (Fig. 1). The division index (cells in cytoplasmic division as a fraction of the total) shows three maxima, a first and a second sharp and third slight separated by deep minima. The times when the first and the second division maxima appear is not much influenced by the washing, but the two last maxima seem perhaps to be lower (0.85–0.52–0.16) than in the proteose-peptone medium (0.85–0.65–0.22) (cf. [12]). The cell number increased 100 per cent with the first, 60 per cent with the second and only slightly with the third maximum.

Respiration intensities.—The respiration per cell of the washed cells (measured 2 hours after the washing) was 25 to 30 per cent of the respiration of the proteose-peptone cells (Table I). This was true for the log-phase cells and for the synchronized cells. Dry matter was determined independently of the respiration experiments and only in the proteose-peptone cells. In theory the data can be compared on a per dry weight basis only if correction is made for the metabolic loss in dry weight suffered by the cells in inorganic medium, prior to the time when the respiration was measured. However, this loss is probably of the order of 5 per cent, thus negligible. There seems to be no difference in the respiration rate per unit dry weight in the normal log phase and in the synchronized cells, irrespective of whether comparison is made in proteose-peptone or in inorganic medium.

The effect of 2,4-DNP on the respiration of Tetrahymena cells.—In the washed log-phase cells the respiration is continuously decreasing. A typical experiment is shown in Fig. 2. The upper curves represent cumulative oxygen uptakes in inorganic medium (pH = 7.3) with no DNP (I*a*) and with DNP added at the time indicated by the arrow, to make the concentrations 1×10^{-4} (II*a*), 3×10^{-4} (III*a*) and 5×10^{-4} (IV*a*) *M*.

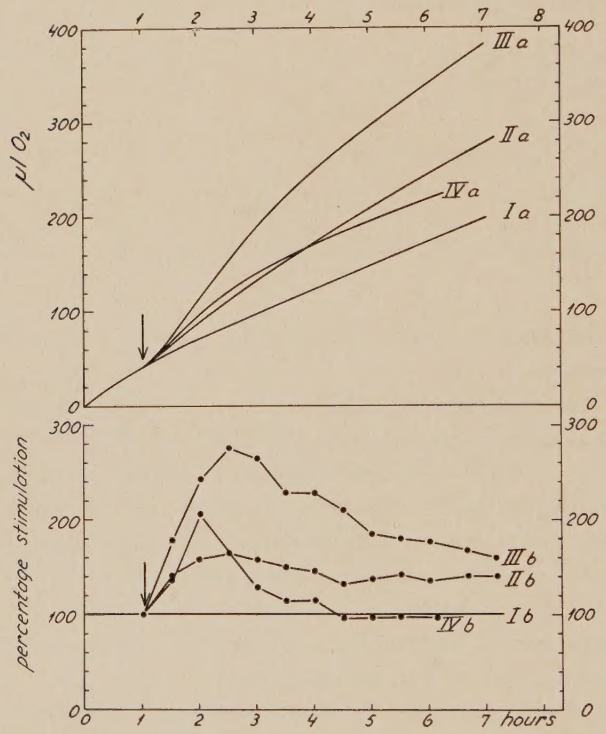

Fig. 2.—DNP added to a washed log phase culture (at the time indicated by the arrow) to make the concentrations: I: 0.0, II: 1×10^{-4}, III: 3×10^{-4} and IV: 5×10^{-4} *M*. The upper curves are cumulative, and the lower are rate curves. (pH 7.3, $t = 28°C$.)

The lower curves (I*b*, II*b*, III*b*, and IV*b*) are rate curves derived from the upper curves. The percentage stimulation or inhibition is plotted against time. For each new time interval the slope of the control curve is put equal to 100 per cent. In this way correction is made for the changing control respiration.

For the concentrations of DNP which are most effective in increasing respiration, the maximal effect is usually in the second hour after addition of the drug. For lower or higher concentrations of DNP the time for maximum stimulation is earlier, rather in the second half hour after addition. Later in the experiment stimulations tend to be lower, and in the case of the higher

concentrations of DNP the initially observed stimulation may turn into an inhibition.

Because for each concentration there is a new time factor involved, no single curve gives the true relation between the concentration of DNP and

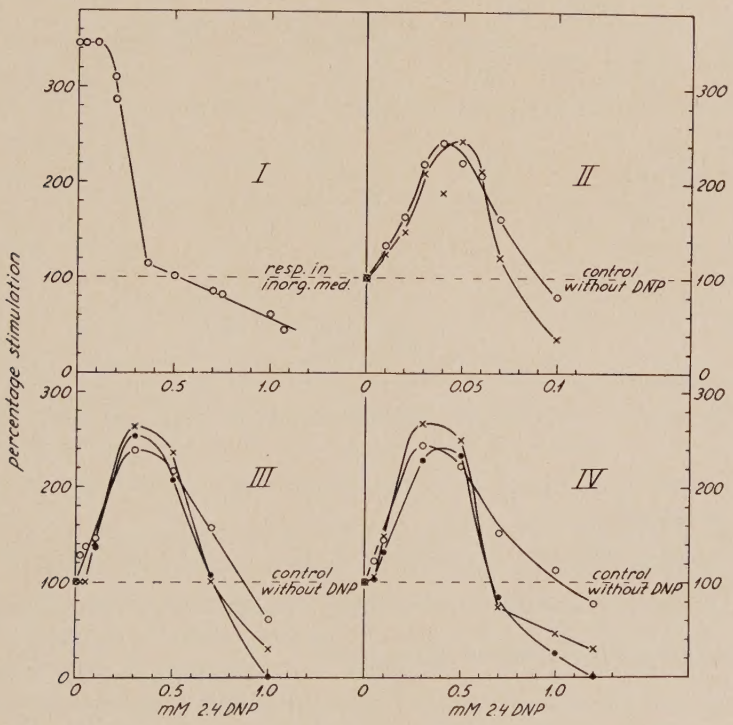

Fig. 3.—I, log-phase cells, proteose-peptone pH 7.0 ± 0.1; II, log-phase cells, inorganic medium pH 6.2 ± 0.1; III, log-phase cells, inorganic medium pH 7.2 ± 0.1; and IV, synchronized cells, inorganic medium pH 7.2 ± 0.1. The curves I–IV show the effect of graded concentrations of DNP on respiration of *Tetrahymena*, measured in the case of the washed cultures 30–60 (○), 60–120 (×) and 120–180 (•) min. after the addition of the drug. In the proteose-peptone culture the rates of respiration in the last half hour before, and in the first half hour after the addition of DNP are compared.

the respiratory effect. Therefore, in order to relate concentration of DNP to its influence on respiration we have chosen to present curves for the three periods 30–60, 60–120, and 120–180 minutes after addition of the drug (Fig. 3). However, for our purpose the important thing is that the three curves all show maximal stimulation of respiration for the same concentration of DNP. Maximal stimulation averages 240 per cent of the level observed without DNP. In the inorganic medium the stimulated respiration corre-

TABLE II

Data give respiration in μl O_2/min/10^6 cells.

	Washed cells[a]		Proteose-peptone cells[b]	
	No DNP	DNP, maximum stimulatory	No DNP	
	1	2	3	2:3
log-phase cells	1.4	3.5	4.8	0.73
synchronized cells	4.6	11.5	15.2	0.76

[a] cf. Fig. 3, II–III–IV.
[b] cf. Fig. 3, I.

TABLE III

	pH = 6.0 – 6.2	pH = 7.2 – 7.4
Conc. in *M* for max. stimulation of respiration	4×10^{-5}–5×10^{-5} (2)	3×10^{-4}–4×10^{-4} (8)
Conc. of undiss. DNP, in *M* (calculated)	2.5×10^{-7}–5×10^{-7}	1.5×10^{-7}–3.2×10^{-7}

Values in parentheses give number of experiments.

sponds to a maximum of 73–76 per cent of the respiration of proteose-peptone cells (Table II). This is true for log-phase, as well as for synchronized cells.

In the presence of proteose-peptone all concentrations of DNP are inhibitory (curve I, Fig. 3).

The concentration of DNP which gives maximal stimulation of respiration in washed *Tetrahymena* cells varies with pH (Table III, upper row), but the concentration of undissociated DNP which corresponds to maximal stimulation of respiration is probably not different at pH 6.2 and pH 7.3.

During this investigation we made a search for possible different responses to DNP of the washed synchronized cells, depending on the point in the cell cycle in which DNP was added. Thus far, we have not been able to demonstrate such differences.

TABLE IV

pH init.	pH final	M DNP final conc.	M DNP undiss. conc.[a]	Time for DNP addition (min. before 1st div. in contr.)	Divisions in % of control 1st div.	2nd div.	later, at end of exp.	DNP treatment (hours)	No. of exp.
6.20		1×10^{-5}	0.8×10^{-7}	45	100	100	100	$5\frac{1}{2}$	1
6.35		3×10^{-5}	1.8×10^{-7}	45	0	0	0	$5\frac{1}{2}$	1
7.25		1×10^{-4}	0.7×10^{-7}	50	100	10	67	5	1
7.30	7.50	1×10^{-4}	0.7×10^{-7}	30–40			80	5–8	6[b]
7.30	7.60	2×10^{-4}	1.3×10^{-7}	30–40			0	5–8	3[b]
7.35		3×10^{-4}	1.7×10^{-7}	50	0	0	0	5	1
7.30	7.60	3×10^{-4}	1.9×10^{-7}	30–40			0	5–8	3[b]

[a] Calculated on basis of initial pH.
[b] These experiments were conducted in Warburg chambers and combined with respiration measurements.

The effect of continuous exposure to 2,4 DNP on cell division in synchronized, washed Tetrahymena cells.—For each respiration experiment with synchronized cells the initial number of cells per ml is known. At the end of the experiment the cells in the respiration chambers were counted, and the number of divisions per original cell was calculated. Table IV shows these results expressed as percentage of the control. In Table IV is included some independent experiments in which the cell number was followed throughout the experiment. It will be seen that the sharpest decrease in cell multiplication is when the DNP concentration increases from 1×10^{-5} to 3×10^{-5} *M* at pH 6.3 and from 1×10^{-4} to 2×10^{-4} *M* at pH 7.3. This should be compared with the finding that maximal stimulation of respiration by DNP, measured in the second hour of the experiment occurs at 3×10^{-5} *M* (pH 6.2) and 3×10^{-4} *M* (at pH 7.3). These observations suggest the strongest interference with cell division by concentrations of DNP which are slightly lower than those which give maximum stimulation of respiration (cf. Tables III and IV). Furthermore, the undissociated DNP seems to be the physiologically active compound interfering with cell division as well as with respiration.

The effect of short exposure to 2,4-DNP on cell division in washed, synchronized Tetrahymena cells.—Cells which were treated in the standard way

to produce synchronization on the inorganic medium were exposed at various ages, to 5×10^{-5} *M* DNP (pH 6.0). They were in contact with the drug for a standard period of 20 minutes, and were then transferred to inorganic medium by 3 washings in the hand centrifuge (total 5 minutes). We always observed that the division which followed the exposure was delayed, but the time interval between this division and the next was not

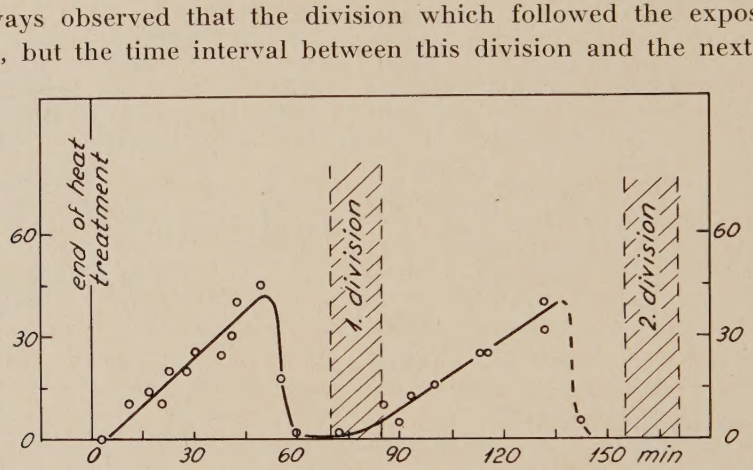

Fig. 4.—Synchronized cells treated for 20 min. with 5×10^{-5} *M* DNP at pH 6.0, $t = 28°C$. For further explanation see the text.

influenced. Fig. 4 shows this delay. For cells treated in the time interval 0–80 minutes the delay is for division 1, and for cells exposed later than this the delay is for division 2. The abscissa gives the time when the cells were immersed in the inhibitor.

During the first 50 minutes or so after heat treatment, or after division, there is a gradual development of sensitivity to DNP. Around 50 minutes, and at 135 minutes, there are sharp decreases followed by low sensitivities for about 25 minutes before the first (at 75 min.) and second (at 160 min.) division maxima.

Exposure to DNP delays all stages by the time exposed or longer. Cells which have just been returned to 28°, just-divided cells, and cells which would normally have divided within 25 minutes have thus far been found to be delayed by 5×10^{-5} *M* DNP only for the time exposed. They are thus blocked while in contact with DNP (however, the concentration is critical; e.g., cells exposed to 3×10^{-5} *M* DNP (pH 6.0) within 20 minutes of division will divide in 1–2 hours when in contact with the inhibitor). Cells in other stages are delayed in division more than the time they have been exposed to DNP, 3×10^{-5} *M*. They are thus set back in time, and how much is in-

dicated by the ordinate readings of Fig. 4. The delay is greater the farther the cells have advanced toward the critical point at 50 minutes after heat treatment or division. In the discussion this will be interpreted as evidence of the gradual accumulation of a store of DNP-labile substances which is depleted or made unavailable around 25 minutes before each division. Unpublished experiments (by L. Rasmussen) indicate that throughout the cell cycle there are variations in the sensitivity to lack of oxygen, similar to those found for DNP.

DISCUSSION

If a population of small cells (log-phase cells from a shaker flask) is transferred to the inorganic medium only those cells which are mature for division will divide. On the other hand, the large [10, 14] stationary-phase cells (from test-tube cultures) are capable of about one division after replacement of the proteose-peptone with the inorganic medium.

This investigation showed that *Tetrahymena* cells which were exposed to synchronizing heat treatment while grown in 2 per cent proteose-peptone will go through two or more divisions after they have been transferred to an inorganic medium. The ability to continue dividing in the absence of growth may be attributed to the unusually large size of the synchronized cells. These cells probably carry stores of a great variety of structural and chemical entities, including deoxyribose nucleic acid (cf. [15, 2]).

In *Tetrahymena* two fractions of the respiration may be distinguished. A fraction of 30 per cent relies on endogenous reserves and can fully support cell division. Another (70 per cent) depends on exogenous supplies and is coupled with growth. Studies on the influence of varying concentrations of urethane on respiration and cell division in *Tetrahymena* led Ormsbee and Fisher [9] to suggest the presence of two independent, parallel respiratory systems. Similarly [4] in the *Arbacia* egg there is an "activity" system of 40 per cent of the respiration to which cell division is coupled. The remaining part of the respiration is a "resting" system.

The respiratory response of washed *Tetrahymena* cells (log-phase and synchronized) to graded concentrations of DNP shows some resemblance to what has been observed for the sea urchin egg (reviews by Clowes [1] and by Krahl [6]) and for yeast [3]. With increasing concentration of DNP the respiration is stimulated to reach a maximum, and with further rise in the level of DNP there is less stimulation and actual inhibition. However, the range of DNP concentrations over which we obtain this graded response is

much narrower than in the case of the sea urchin egg. In *Tetrahymena* the lowest concentration of DNP which gives stimulation (pH 7.3) is 1×10^{-4} *M*, and 1×10^{-3} *M* produces inhibition. In the *Arbacia* egg (pH 8.2) the corresponding concentrations are 1×10^{-6} *M* and 1×10^{-3} *M* [7]. Experiments in which extracellular pH was varied suggest that in *Tetrahymena* maximal stimulation of respiration and inhibition of cell division correlated much better with undissociated than with total DNP. When DNP is increased (pH 7.2) from 1×10^{-4} to 2×10^{-4} *M* the cell divisions are strongly influenced. In this concentration range the respiration is progressively stimulated. DNP uncouples respiration and phosphorylation [8, 5]. The results presented supply evidence that in *Tetrahymena*, as in other cells, division requires phosphorylation.

Experiments with short exposures to DNP suggest that oxidative phosphorylation in preparation for division occurs in all phases of the cycle. The sensitivity curves to DNP (Fig. 4) and to oxygen lack (Rasmussen, unpublished) suggest that all such preparation, achieved in the first 50 minutes after end of heat treatment, or during the same period of time after the first division, is reversed by exposure to DNP (5×10^{-5} *M*, pH 6.0) for 20 minutes, or to lack of oxygen for 10–15 minutes. Our data show that when exposure is made within the last 25 minutes before a division, there is no reversal but only a block of the oxidative phosphorylation which during the period of exposure would have permitted the cell to complete its preparation for division. In terms of Swann's view [13] concerning the sea urchin egg this would mean that at 50 minutes phosphate esters, previously accumulated, split to make the later division possible. A second possibility is that there is transfer of ~P to esters which are insensitive to DNP and to lack of oxygen; a third is that at 50 minutes the ~P of esters are bound to the cell structure so that they are no longer available for purposes other than cell division.

SUMMARY

It is possible to maintain the synchronous division of *Tetrahymena pyriformis* on an inorganic medium, and thus on endogenous sources. The oxygen uptake for synchronized and normal log-phase cells is compared in proteose-peptone and inorganic media. The effects of 2,4-dinitrophenol on respiration and cell division in synchronized and normal log-phase cells are demonstrated and discussed.

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Experimental Cell Research **13**, *454–474 (1957)*

ANALYSIS OF THE SURFACE STRUCTURES OF THE SEA URCHIN EGG BY MEANS OF ANTIBODIES[1]

II. THE J- AND A-ANTIGENS

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Received April 2, 1957

When sea urchin eggs are exposed to rabbit antiserum, a number of microscopic changes are induced in the eggs. The most important changes described thus far are (1) a precipitation of the jelly layer of the egg, (2) a parthenogenetic activation of the unfertilized egg, and (3) various disturbances of fertilization [14, 15, 16, 18, 19].

The species and tissue specificity of the reactions has been demonstrated in previous publications. Various factors determining the response of the eggs have been studied in some detail. The relationship between the dose of the antiserum and the response of the eggs has been established for the reactions mentioned above, which therefore can be studied quantitatively. Quantitative studies have thus led to certain conclusions concerning the minimum number of antigenic factors present in the surface of the egg [16].

In a study of cell structures with the aid of antisera, inhibition techniques stand out as powerful analytical tools. Thus, antibodies can be removed from the antisera by means of absorption with extracts from the cells. When the absorbed antisera are added to the cells, one or several of the normal antiserum activities may be inhibited. Hence, a study of the inhibitory capacity of extracts subjected to various treatments would permit definite conclusions concerning the number and the chemical nature of the antigens involved in the response reactions of the cells.

In this paper the results of an investigation of this kind will be presented. The activities of the antisera which will be considered are the precipitation of the jelly layers and the parthenogenetic activation of the unfertilized egg of the sea urchin *Paracentrotus lividus*. The effects of antiserum on fertilization will be described in a following paper.

[1] Supported by grants from the Swedish Natural Science Research Council and from the Hierta-Retzius' Foundation of the Swedish Academy of Science.

MATERIAL AND METHODS

Antisera.—The rabbit antisera against unfertilized, jelly free eggs, jelly substance and sperm of the sea urchin *Paracentrotus lividus* and the normal sera for the controls were the same as those described in the preceding paper of this series [16].

Preparation of antigens for the absorption of antiserum.—The antisera were absorbed (1) with homogenates of jelly free, unfertilized *Paracentrotus* eggs, (2) with egg extracts, (3) with isolated jelly substances, or (4) with sperm homogenate. Preparations of the same type, obtained from eggs of *Psammechinus microtuberculatus* or *Arbacia lixula* were used as controls.

The *eggs* were freed from their jelly layers at pH 5.2 [31]. The concentrated egg suspensions were made up to volume and aliquots were taken for the determination of the number of eggs in the suspension [16]. The samples were dialyzed against distilled water in the cold and homogenized in a motor-driven glass homogenizer. Further treatments will be described below. For the absorption of the antisera the samples were dialyzed against sea water. Aliquots were taken for nitrogen determination at the appropriate steps during the fractionation procedure. The number of eggs used for the absorption could be deduced from these nitrogen values.

The *jelly substance* of the eggs was isolated as described previously. After isolation the material was adjusted to pH 8 by the addition of 0.1 *M* NaOH. One sample was concentrated by pervaporation; two samples were purified by precipitation with ethanol [31]. Prior to absorption the samples were dialyzed against sea water. The content of fucose in these antigen solutions was found to be useful for their standardization.

The *sperm* (*Paracentrotus*) was washed by repeated centrifugation and resuspension in sea water, homogenized in distilled water and finally lyophilized. For absorption the lyophilized powder was suspended in sea water.

Precipitin tests. –The content of soluble antigens in the solutions was tested by means of double diffusion in a saline agar gel [13, 17]. Prior to use, the antigen solutions were centrifuged and both antigens and antisera were dialyzed against 0.9 per cent saline.

Antiserum absorption.—Two procedures were used throughout: (1) aliquots of 0.1 ml of dilution series of the antigens were mixed with aliquots of one ml of the antisera, or (2) 0.025, 0.05, 0.1, 0.2 ... ml respectively of the appropriately diluted antigen solutions were added to aliquots of one ml of the antisera.[1] The changes which occurred in the total volume of the absorbed mixtures when the second procedure was employed were disregarded. However, this method had the advantage that the activity of the absorbed antisera could be directly compared with that of antisera diluted with corresponding volumes of sea water. The absorbed antisera were left at room temperature for several hours and were centrifuged after 12 hours in the refrigerator. They were kept frozen until use.

In addition to the antisera diluted with sea water, normal sera diluted with antigen were used for control.

[1] The figures show the proportions in which the reagents were mixed. Actually, larger amounts were sometimes employed, since each absorbed sample had to be used for several experiments.

Inhibition tests.—The jelly-precipitating and parthenogenetic effects of absorbed and unabsorbed antisera were studied in experiments performed as described previously [15, 16]. For the present purpose only highly reactive eggs could be used. Hence, the eggs from 20 30 sea urchin females were tested individually in preliminary experiments by mixing three drops of unabsorbed antiserum with one drop of sea water containing ~ 200 eggs. The samples were observed in the microscope after 20–30 minutes. The egg from a well responding batch were then quickly washed and aliquots of 0.2 ml sea water containing ~500 eggs were mixed with aliquots of 0.8 ml of the absorbed or unabsorbed antisera. The time between the isolation of the eggs from the sea urchins and the start of the final experiment did not exceed one hour.

Chemical analyses of the antigen preparations.—Nitrogen was estimated by a micro-Kjeldahl procedure [3] and the content of ribonucleic acid was determined by measuring the absorption in the ultraviolet at 260 mμ. The fucose content of the jelly preparations was estimated according to Dische and Shettles [4]. For analysis, the jelly preparations were handled as described by Vasseur [32]. The content of hexoses in the egg preparations was determined by the cysteine sulfuric acid procedure of Dische *et al.* [5]. For paper chromatography of the sugars Munktell OB paper was used with *n*-butanol:water (6:1), *n*-butanol:ethanol:water (4:1:2), or phenol:water [7] as solvents, and $AgNO_3$ [26] or anilin phthalate [12] as developers. For analysis, the samples were concentrated by evaporation *in vacuo* at 50°C and hydrolyzed with 1 *N* H_2SO_4 on a boiling water bath for 5 hours. Ions were removed by passing the neutralized hydrolysates through columns of Dowex 50 in the H^+ form and Dowex 2 in the acetate form. In samples of a volume of one ml or less, neutralization was omitted. The eluates of the ion exchange columns were then concentrated by evaporation. The amount of acid hydrolyzable sulfate was determined with benzidin [6] after hydrolysis of the antigen solutions with 0.5 *M* HCl for 1.5 hours at 2.3 at. Electrophoretic analyses were carried out in a Spinco electrophoresis apparatus or in that of Antweiler.

INHIBITORY CAPACITIES OF JELLY SUBSTANCE AND OF EGG HOMOGENATE

The antibodies which precipitate the jelly layers of the *Paracentrotus* eggs differ from those which lead to parthenogenetic activation [16]. Moreover, whereas the antigen responsible for the jelly-precipitating antibodies is located mainly in the jelly layer, the distribution of the antigen(s) causing formation of the activating antibodies appears to be more variable. The validity of these conclusions was tested in a series of inhibition experiments.

A number of antigen solutions, used for absorption, are described in Table I. The number of eggs present in the solutions PE-I and PE-II was estimated from the nitrogen analyses; 10 12 μg of *non-dialysable* nitrogen corresponded to ~ 1000 eggs. Whereas these values are highly reproducible, the amount of jelly fucose equivalent to a certain number of eggs is much more variable.

TABLE I. *Various egg homogenates and jelly preparations used in the inhibition experiments illustrated in Figs. 1 and 2.*

The various samples were each taken from different egg batches. The amounts given in the last column refer to the concentrations of the solutions as used in the inhibition experiments, illustrated in Figs. 1 and 2. For details of preparation cf. text.

Sample	Nature of sample	Amount per ml solution
PE-I	Unfertilized, jelly free *Paracentrotus* eggs, frozen	1.12 mg undial. nitrogen (125,000 eggs)
PE-II	Unfertilized, jelly free *Paracentrotus* eggs, lyophilized	0.279 mg undial. nitrogen (32,000 eggs)
PsE	Unfertilized, jelly free *Psammechinus* eggs, frozen	1.80 mg undial. nitrogen (egg-number unknown)
PJ-I	*Paracentrotus* egg jelly, purified with alcohol, frozen	0.296 mg fucose (~20,000 eggs)
PJ-II	*Paracentrotus* egg jelly, neutralized, frozen	0.153 mg fucose (~10,000 eggs)
PJ-III	*Paracentrotus* egg jelly, pervaporated, lyophilized	0.340 mg fucose (~23,000 eggs)
PJ-IV	*Paracentrotus* egg jelly, purified with alcohol, lyophilized	0.102 mg fucose (~7,000 eggs)

The numbers in Table I are deduced from one sample in which 15 μg fucose corresponded to ~ 1000 eggs. This figure represents the highest value found; the average amount of jelly fucose per egg was lower.

Figs. 1 *a* and 2 *a* show the inhibiting effect of the antigen preparations on the jelly-precipitating activity of the two anti-jelly sera J-3 and J-4, respectively. The number of visibly jelly-precipitated eggs is used as an index of the antiserum action. The reduction in the number of jelly-precipitated eggs obtained in an inhibition experiment is paralleled by a reduction in the degree of agglutination and by a reduction of jelly contraction [16].

Apparently, the inhibitory capacity of the four jelly preparations was considerable; about 15 μg fucose was sufficient in most cases for a 50 per cent inhibition. This amount corresponds to the total jelly substance of, roughly, 1000 eggs or more. These values, although approximate, show the marked differences in the inhibiting capacity of the jelly preparations in contrast to that of the egg homogenates. The inhibition obtained with

~100,000 eggs of PE-I, illustrated in Fig. 1*a*, was hardly significant. No inhibition was obtained with a corresponding amount of PE-II in the experiment shown in Fig. 2*a*.

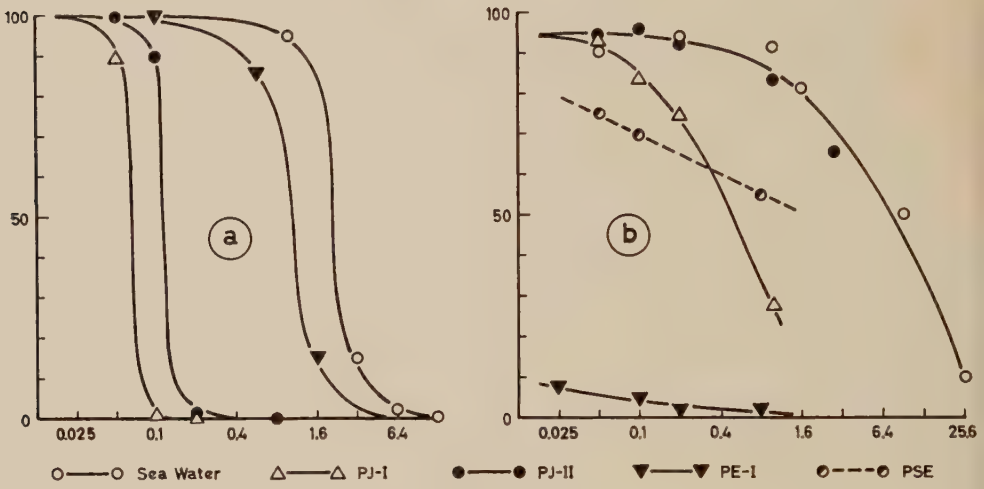

Fig. 1.

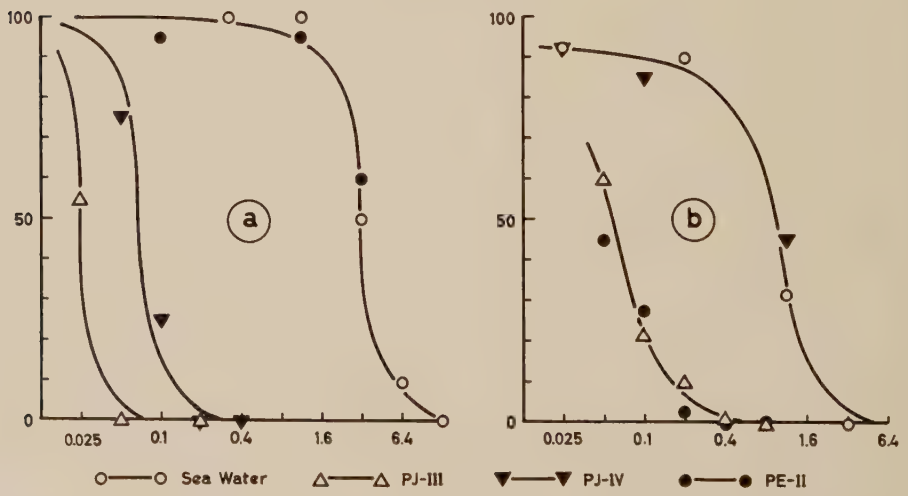

Fig. 2.

Figs. 1 and 2.—500 eggs in 0.2 ml sea water, mixed with aliquots of 0.8 ml antiserum, absorbed, or diluted with sea water. Eggs from different females were used for each of the 4 experiments. *Abscissae*, ml of various antigen solutions used for absorption of one-ml aliquots of anti-jelly serum J-3 (1*a*) or J-4 (2*a*) or of anti-egg serum P-10 (1*b*) or P-11 (2*b*). For explanation of symbols cf. Table I. *Ordinates*, per cent jelly-precipitated eggs (1*a* and 2*a*) or per cent activated eggs (1*b* and 2*b*). Each symbol represents 200 counted eggs.

The unfertilized sea urchin egg contains a number of jelly-precipitating substances [21, 27]. Therefore exposure of the eggs in an inhibition experiment to the absorbed antiserum, containing egg homogenate, may lead to an "unspecific" jelly precipitation. This jelly precipitation might be quite unrelated to that induced by the antibodies. The data in Figs. 1*a* and 2*a* show that this was not the case; actually, the relative number of jelly precipitated eggs decreases with increasing "dilution" (i.e., absorption) of the sera with the homogenates. It could also be ascertained that exposure of the eggs to mixtures of normal serum and homogenates does not cause jelly precipitation.

Absorption of the anti-jelly sera with large amounts of jelly substance from *Psammechinus microtuberculatus* had only a slight inhibitory effect. This is in agreement with earlier findings in which a high degree of species specificity of the antiserum activity was demonstrated [15].

In the experiment illustrated in Fig. 1*b*, ~30 μg of non-dialyzable nitrogen of the fresh preparation PE-I corresponding to ~3000 eggs caused nearly complete inhibition of the parthenogenetic activity of antiserum P-10. In the experiment of Fig. 2*b*, ~15 μg of non-dialyzable nitrogen (~1500 eggs) of the lyophilized preparation PE-II were sufficient for a 50 per cent inhibition of the activity of P-11.

As shown by the typical data, obtained with *Psammechinus* homogenate (Fig. 1*b*), absorption with homogenates from other species than *Paracentrotus* has much less effect. This demonstrates that the inhibiton is due to the elimination from the antisera of activating antibodies.

The inhibitory capacity of the jelly preparations was much more variable than that of the egg homogenates. PJ-II had no measurable effect. PJ-I had an inhibitory action, although as much as 150 μg of fucose were needed for 50 per cent inhibition (Fig. 1*b*). In the experiment shown in Fig. 2*b* one of the preparations was without effect, whereas the other one inhibited strongly. Here, only 20 μg fucose (~1300 eggs or more) were needed to attain a 50 per cent inhibition. The variability in the inhibiting capacity of the jelly preparations is in agreement with the variability found earlier in the parthenogenetic potency of various anti-jelly sera [16].

In Table II the results are given of an experiment in which the inhibiting effect of the antigen preparations on the jelly-precipitating and activating action of an antiserum were tested simultaneously. The eggs used in this experiment were from a batch giving both responses, and the antiserum was the anti-jelly serum J-4 exhibiting both activities [16]. The results indicate that different antigen-antibody reactions are responsible for the two activities

TABLE II. *Inhibition of jelly precipitating and of activating actions of anti-jelly serum J-4.*

Aliquots of one ml of antiserum or normal serum, respectively, absorbed with aliquots of 0.1 ml of various egg homogenates or jelly preparations. The samples used for absorption were taken from the stock solutions of the preparations described in Table I. In column 2 below are given the amounts actually added to the sera. For details of preparation cf. text.

Eggs from the same female were used throughout. Aliquots of 0.2 ml sea water, containing 500 eggs, were mixed with 0.8 ml of absorbed or unabsorbed antisera or normal sera, respectively. Incubation for the study of jelly precipitation and activation as previously described. Results based on counts of 200 eggs in each case.

C = normal serum.

Sample	Amount antigen per ml antiserum	Per cent affected eggs: Jelly precipitation J-4	Jelly precipitation C	Activation J-4	Activation C
Sea water	0.1 ml	100	0	44	0
Jelly PJ-I	1,000 eggs	75	0	36	0
	20,000 eggs	0	0	12	0
Egg homogenate PE-II	2,000 eggs	100	0	3	0
	20,000 eggs	100	0	0	0
Paracentrotus sperm, washed	40 μg N	100	0?	50	0
	400 μg N	100	100	42	0
	4,000 μg N	100	100	49	0

of the antiserum. Table II also shows that the absorption of antiserum with considerable amounts of sperm homogenate did not inhibit activation. The effect of the sperm on the jelly precipitation could not be tested owing to the strong jelly-precipitating action exerted by the sperm homogenate [21, 27] present in the reaction mixture.

JELLY PRECIPITATION: THE J-ANTIGEN

In the following, the antigen involved in jelly precipitation will be called the *J-antigen* and the corresponding antibodies the J-antibodies. The results reported in this paragraph have some bearing on the question of the chemical nature of this antigen.

The jelly layer of the sea urchin egg contains carbohydrate, amino acids and sulfate as its main components [20, 23, 29]. While the carbohydrate and sulfate content of the isolated jelly substance is high (45 and 25 per cent respectively), the nitrogen content is low (2–6 per cent) and varies with the mode of preparation [27, 31]. No hexosamines or hexuronic acids have thus far been found [31, 28]. The

jelly polysaccharide consists of one or sometimes two types of monosaccharides. These differ in different species, although L-fucose predominates in many. The jelly layer of *Paracentrotus lividus* consists of fucose and glucose, with fucose occurring in large excess. The substances show a very fast anodic migration in the electrophoresis apparatus and this migration changes only slightly within pH 2–8. Tyler assumes [30] that the jelly layer of the sea urchin egg is a homogenous substance, consisting of elongated molecules of a molecular weight of about 300,000. The nitrogenous part, i.e. the amino acids or the proteins, and the polysaccharide are interlinked very firmly [31, 28] but the types of linkage are not known with certainty. The only attempt, thus far, to study the structure of the polysaccharide has been made by Vasseur. On the basis of oxidation studies with periodate [32] Vasseur concluded that the jelly polysaccharide of the sea urchin *Echinocardium cordatum* is composed of a main chain of fucose-sulfate residues, each carrying another fucose-sulfate residue as a side chain.

It was of interest to study whether the J-specificity of the jelly preparations is bound to the polysaccharide or to the amino acid part. A series of studies, as yet incomplete, suggests a close correlation between the J-inhibiting

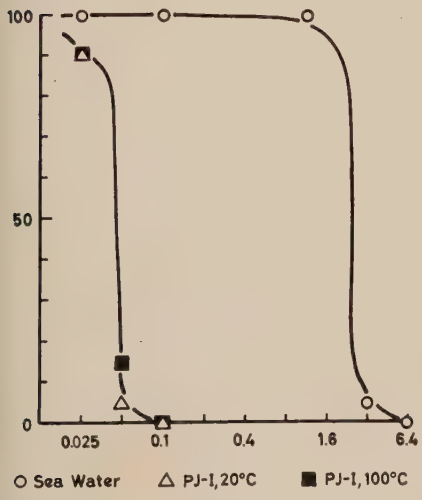

Fig. 3.

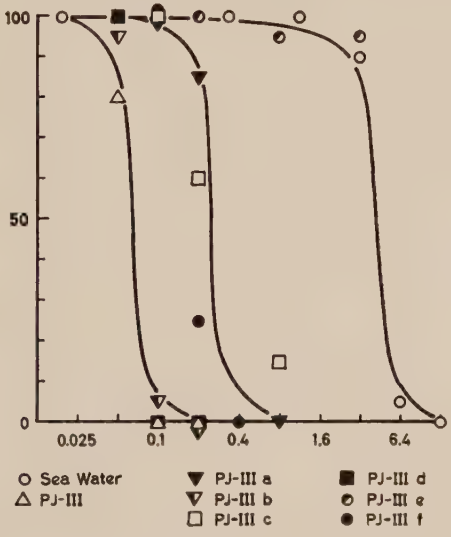

Fig. 4.

Fig. 3.—500 eggs in 0.2 ml sea water, mixed with aliquots of 0.8 ml of anti-jelly serum J-3, absorbed, or diluted with sea water. *Abscissa*, ml of various antigen solutions used for absorption of one-ml aliquots of antiserum. For explanation of symbols cf. text. *Ordinate*, per cent jelly precipitated eggs. Each symbol represents 200 counted eggs.

Fig. 4.—500 eggs in 0.2 ml sea water, mixed with aliquots of 0.8 ml of anti-jelly serum J-3, absorbed, or diluted with sea water. *Abscissa*, ml of various antigen solutions used for absorption of one-ml aliquots of antiserum. For explanation of symbols cf. Table III. *Ordinate*, per cent jelly-precipitated eggs. Each symbol represents 200 counted eggs.

TABLE III. *Various treatments of a jelly preparation and an egg homogenate of* Paracentrotus lividus *for absorption of antiserum.*

PJ-III: Pervaporated and lyophylized jelly substance. Aliquots of 2 ml of a solution were used for incubation.

PE-II: Unfertilized and lyophilized jelly free eggs. The homogenate was heated on a boiling water bath for 60 minutes, centrifuged and dialyzed. Aliquots of 3 ml of the extract were used for incubation.

Oxidation of the samples with a 0.01 *M* solution of periodate in a HCl-biphtalate buffer of pH 3.5. Acid hydrolysis with 1 *M* HCl. After incubation the solutions were dialyzed.

Sample	Added to sample for incubation	Incubation (240 min.)	Amount/ml final solution	
			μg N	μg fucose
PJ-III	+2 ml H_2O	20° C	27	295
PJ-III a	+1 ml buffer pH 3.5 + 0.2ml $NaIO_4$ + 0.8 ml H_2O	50 °C	31	
PJ-III b	+1 ml buffer pH 3.5 + 1 ml H_2O	50° C	29	
PJ-III c	+1 ml buffer pH 3.5 + 1 ml H_2O	100° C	23	
PJ-III d	+1 ml buffer pH 3.5 + 1 ml H_2O	20° C	31	298
PJ-III e	+2 ml (H_2O + HCl → pH 1)	100° C	26	53
PJ-III f	+2 ml (H_2O + HCl → pH 1)	20° C	32	300
PE-II	+7 ml H_2O	20° C	48	
PE-II a	+3 ml buffer pH 3.5 + 0.5 ml $NaIO_4$ + 3.5 ml H_2O	50° C	14	
PE-II b	+3 ml buffer pH 3.5 + 4 ml H_2O	50° C	15	
PE-II c	+3 ml buffer pH 3.5 + 4 ml H_2O	100° C	16	
PE-II e	+7 ml (H_2O + HCl → pH 1)	100° C	24	
PE-II f	+7 ml (H_2O + HCl → pH 1)	20°C	24	

capacity and the fucose content of the preparations. The data shown in the Figs. 1*a* and 2*a* also point in that direction. In any case, the J-inhibiting capacity of the preparations could not be correlated with their content of non-dialyzable nitrogen. The ratio of fucose to nitrogen in the preparations was highly variable.

The assumption that the J-specificity is bound to the polysaccharide is supported by its stability when heated to 100°C at a pH close to neutrality. Fig. 3 gives the results of a typical experiment, using for absorption two aliquots of the jelly preparation PJ-I (Table I). One of them was heated for 240 minutes on a boiling water bath at pH 7.9. As can be seen, the inhibitory capacity of the treated sample was indistinguishable from that of the untreated.

The antigenic specificity of polysaccharides can be studied by means of mild acid hydrolysis [9, 25]. Oxidation with periodate has been used for

the same purpose [9, 1, 25]. Therefore, it was of interest to study what influence such treatments would have on the J-specificity of the jelly preparations. In the upper part of Table III, various treatments of this kind are described. The results obtained with the treated solutions in an inhibiton experiment are given in Fig. 4. As can be seen, treatment at pH 3.5 both at 20°C and at 50°C did not alter the inhibitory capacity of the solutions as compared with that of the untreated control. Heating to 100°C at pH 3.5 or treatment with periodate led to a significant reduction of this capacity, although it should be noted that the reduction was not total in either case. For the periodate treatment the incomplete reduction may be ascribed to the unfavourable periodate: fucose ratio prevailing in this experiment. A certain reduction of the inhibitory capacity was also observed in the solution treated with HCl at pH 1 and 20°C. Four hours' treatment at pH 1 and 100°C apparently destroyed the J-antigen.

The results of this experiment present further evidence for the carbohydrate nature of the J-antigen. From Table III it can also be seen that there was no obvious correlation between the nitrogen content of the treated solutions and their inhibitory capacity. The modes of action of the periodate and of the treatment with acid have not yet been studied and the analytical data of the changes in the carbohydrates upon these treatments are thus far incomplete. Hence, at present nothing can be said about the fine structure of the J-antigenic determinants. The possible role of the sulfate groups in this connection is also unknown. Studies along these lines are in progress.

PARTHENOGENETIC ACTIVATION: THE A-ANTIGEN

Thus far, only indirect evidence has been produced for the specificity of the antigen-antibody reaction, involved in the parthenogenetic action of the antisera [15, 16]. The experiments described in the following were undertaken in order to throw more light on this question.

A series of inhibition experiments, using antisera absorbed with cell fractions, indicated that most of the inhibitory activity was bound to a particulate fraction of the *Paracentrotus* egg. This fraction could be separated from the egg homogenate by centrifugation at low speed. However, the inhibitory principle of the preparations was readily brought into solution with distilled water, independent of whether the uncentrifuged homogenate or a particulate fraction was used for extraction; thus, in the inhibition test, a centrifuged saline extract obtained from the total egg homogenate had the same inhibitory capacity as a corresponding amount of the uncentrifuged homogenate.

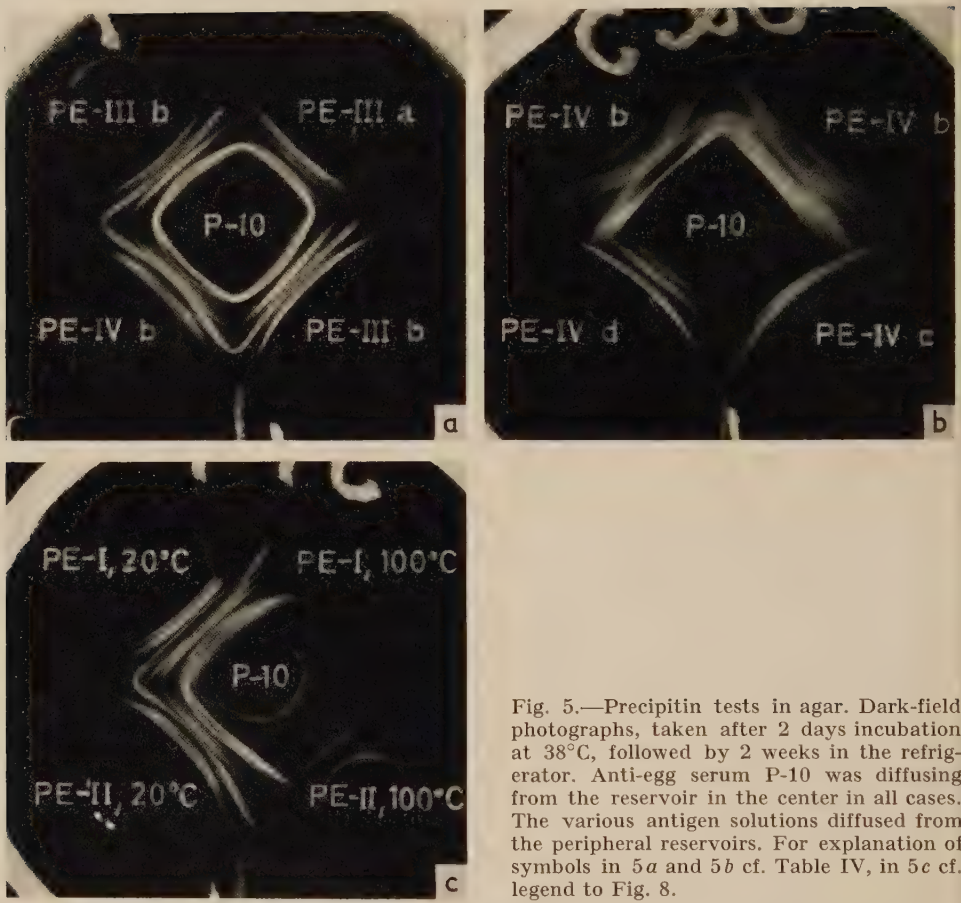

Fig. 5.—Precipitin tests in agar. Dark-field photographs, taken after 2 days incubation at 38°C, followed by 2 weeks in the refrigerator. Anti-egg serum P-10 was diffusing from the reservoir in the center in all cases. The various antigen solutions diffused from the peripheral reservoirs. For explanation of symbols in 5*a* and 5*b* cf. Table IV, in 5*c* cf. legend to Fig. 8.

A water extract of the unfertilized eggs contains a large number of antigenically and chemically different proteins; antibodies against these occur in the anti-egg sera [13; 16, Fig. 9]. It was interesting to establish whether or not the reactions between some of these antigens and their antibodies are involved in the parthenogenetic action of the antisera. In order to eliminate at least part of these antigens, the homogenates can be treated with proteolytic enzymes. In Table IV, the treatments of egg homogenates with trypsin and pepsin, respectively, are described. The treated solutions were then used (1) as antigens in precipitin reactions in agar, and (2) as absorbing solutions in inhibition experiments.

Fig. 5*a* shows that treatment with trypsin removed part of the precipitable antigens present in the untreated control. Owing to this treatment, these

TABLE IV. *Treatment of egg homogenates of* Paracentrotus lividus *with proteolytic enzymes for absorption of antiserum.*

PE-III: Homogenate made from frozen, jelly free eggs; 200,000 eggs/ml stock solution.
PE-IV: Homogenate made from lyophilized, jelly free eggs; 110,000 eggs/ml stock solution.
All samples were dialyzed and centrifuged before use. Aliquots of 3 ml of the extracts were used for the incubation. Trypsin: 0.1 per cent solution. Soybean trypsin inhibitor: 0.15 per cent solution. Buffer: phosphate. Pepsin: 0.1 per cent solution. HCl: 0.1 *M*.
C-a, C-b: Controls containing 3 ml H_2O instead of egg extract.

Sample	Added to sample for incubation	Incubation at 35° C Time in min.	Added after incubation
PE-III a	+3 ml buffer (pH 7.9) +3 ml trypsin	300	1 ml trypsin inhibitor
PE-III b	+3 ml buffer (pH 7.9) +3 ml H_2O	300	1 ml H_2O
PE-III c	+3 ml (H_2O + HCl → pH2) +3 ml pepsin	300	1 ml H_2O
PE-III d	+6 ml (H_2O + HCl → pH 2)	300	1 ml H_2O
PE-IV a	+3 ml buffer (pH 7.9) +3 ml trypsin	600	1 ml H_2O
PE-IV b	+3 ml buffer (pH 7.9) +3 ml H_2O	600	1 ml H_2O
PE-IV c	+3 ml (H_2O + HCl → pH 2) +3 ml pepsin	300	1 ml H_2O
PE-IV d	+6 ml (H_2O + HCl → pH 2)	300	1 ml H_2O
C-a	+3 ml buffer (pH 7.9) +3 ml trypsin	—	1 ml trypsin inhibitor
C-b	+4 ml (H_2O + HCl → pH 2) +3 ml pepsin	—	—

antigens had in fact lost their specificity; they no longer absorbed the corresponding precipitating antibodies from the antiserum when the solutions were tested in absorption experiments. The results were similar when the solutions were treated with pepsin. In this case the lowering of the pH, a prerequisite of the enzyme treatment, in itself removed part of the antigens from the solution (Fig. 5*b*). However, Figs. 5*a* and 5*b* also show that several fractions of the precipitable antigens were not attacked by the treatments. There is evidence that most of these antigens were also proteins.

The results of two independent inhibition experiments with the preparations of Table IV are given in Figs. 6 and 7. The data show that the antigen(s) involved in activation do not belong to the fractions destroyed by the treatment with trypsin or pepsin. Neither did the enzyme treatments lead to liberation of antigens participating in this reaction [9]. The reasons for the slight inhibiting action of the enzyme controls C-a and C-b are not clear; thus, it cannot yet be stated whether this inhibition is due to an action of the enzymes on the eggs or on the antisera.

As a next step, the inhibiting capacity of heated egg homogenates was compared with that of unheated homogenates. In Fig. 8, the data from a typical experiment are given. Even in this case, aliquots of each preparation were tested in the agar test. Fig. 5*c* shows that the heating removed almost all soluble and precipitable antigens; the finding could be confirmed by means of antiserum absorption. In contrast, the heated preparation did not differ

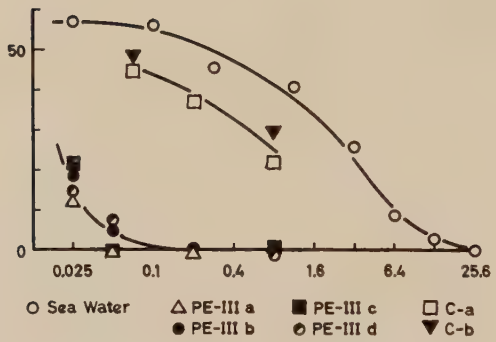

Fig. 6.

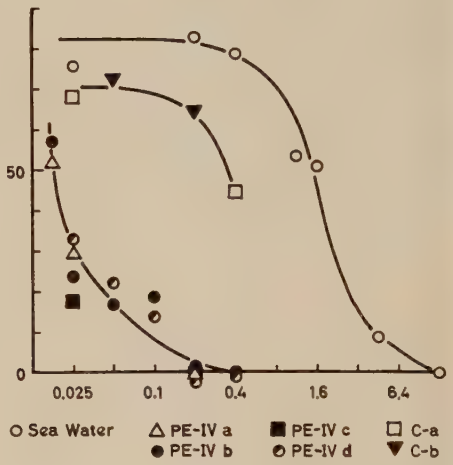

Fig. 7.

Figs. 6 and 7.—500 eggs in 0.2 ml sea water, mixed with aliquots of 0.8 ml of anti-egg serum P-10 (Fig. 6) or P-9 (Fig. 7), absorbed, or diluted with sea water. Eggs from different females were used for each of the 2 experiments. *Abscissae*, ml of various antigen solutions used for absorption of one-ml aliquots of antiserum. For explanation of symbols cf. Table IV. *Ordinates*, per cent activated eggs. Each symbol represents 200 counted eggs.

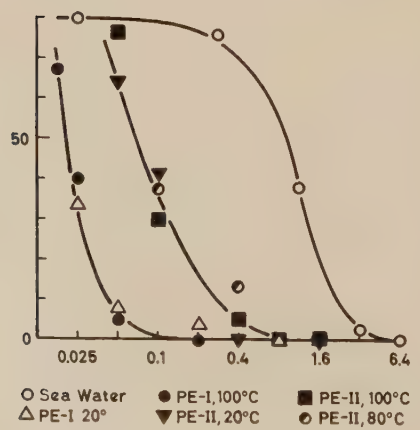

Fig. 8.—500 eggs in 0.2 ml sea water, mixed with aliquots of 0.8 ml of anti-egg serum P-10, absorbed, or diluted with sea water. *Abscissa*, ml of various antigen solutions used for absorption of one-ml aliquots of antiserum. *PE-I* and *PE-II*, egg homogenates described in Table I. *20°C*, untreated samples. *80°C*, and *100°C*, aliquots heated to 80° or 100°C for 4 hours at pH 8. All samples were dialyzed and centrifuged before they were used for absorption. *Ordinate*, per cent activated eggs. Each symbol represents 200 counted eggs.

TABLE V. *Inhibition of activating action of anti-egg serum P-10 with extracts from jelly free eggs of* Paracentrotus lividus (*PE*), Psammechinus microtuberculatus (*PsE*) *and* Arbacia lixula (*AE*).

Egg homogenates were heated on a boiling water bath for 60 minutes, dialyzed against sea water, centrifuged and finally diluted to contain ~10,000 or ~20,000 eggs/ml, respectively. Aliquots of 0.1 ml of the absorbing solutions were added to one ml of antiserum. 500 eggs of *Paracentrotus lividus* in 0.2 ml sea water were mixed with 0.8 ml of absorbed or unabsorbed antisera. Incubation in the cold room for two hours. Observation of the samples 4 hours after mixing. Per cent activated eggs based on counts of 200 eggs in each case.

Sample	Absorption	Per cent activated eggs Female No. 1	2	3
0	0.1 ml sea water	48	72	69
PE	1000 eggs	13	24	30
	2000 eggs	0	5	0
PsE	1000 eggs	25	46	48
	2000 eggs	18	29	30
AE	1000 eggs	50	69	58
	2000 eggs	41	57	49

from the unheated ones when tested in the inhibition experiment (Fig. 8). Confirmative results were obtained with the other activating antisera.

Whereas the inhibition of the jelly-precipitating action of antiserum, described in this paper, can be assumed to be due to the absorption of antibodies, this is not as obvious for the inhibition of the activating action. For example, heating of the egg homogenates may liberate substances which suppress activation when added to the eggs in the inhibition experiment, together with the absorbed antiserum. Other examples of an unspecific inhibitory effect have already been given, viz. the inhibition obtained with the enzyme controls in the experiments illustrated in Figs. 6 and 7.

It may be postulated that the same amount of possible unspecific inhibitors is extracted from the same amount of sea urchin eggs from different species. Hence, a comparison between the inhibiting capacity of extracts obtained from various species and that obtained from *Paracentrotus* will be useful as a control of the specificity of inhibition. This has been illustrated already in Fig. 1 *b* by the data obtained with an homogenate from *Psammechinus microtuberculatus*. In Table V, the results are given of an additional control, in which equal amounts of extracts from three different species were used for

inhibition. The data demonstrate that the inhibition is specific and, hence, it must be caused by the absorption of specific antibodies from the antiserum. The occurrence of cross reactions is not surprising [15].

The results reported here show that the activating action of the antisera is not due to a general and unresolvable stimulation of the eggs by antibodies [11]. Rather, it must be caused by antibodies reacting with a particular antigen which we may call the *A-antigen*. The A-antigen is soluble in water and is heat stable at pH 7–8. It differs from the J-antigen and from the bulk of the soluble protein antigens. It is probably immunologically homogenous, but this remains to be substantiated.

PRELIMINARY EXPERIMENTS ON THE CHEMICAL NATURE OF THE A-ANTIGEN

The content of non-dialyzable nitrogen in the heated egg extracts, described above, was only 20 to 30 per cent of that in the supernatant of the unheated homogenates. The solutions contained considerable amounts of ribonucleic acid split off from the proteins during heating. The content of carbohydrate was also high. One ml heated extract (egg batch PE-I), made from fresh material, contained ~ 230 μg hexose (calculated as glucose), 345 μg ribonucleic acid and 252 μg nitrogen; one ml of the more diluted extract PE-II, made from lyophilized material, contained ~ 110 μg hexose, 122 μg ribonucleic acid and 74 μg nitrogen. Paper chromatography showed in both extracts glucose, mannose and ribose as the only detectable carbohydrates. Attempts to show the presence of hexosamines have thus far been without success.

Fig. 9 shows the results of an electrophoretic analysis of two egg extracts, obtained after heating. Obviously, these heated extracts were still heterogeneous and contained a number of strongly acid components. The mobilities ($\times 10^5$) for the three main components were for 1: -1.59, for 2: -6.15, and for 3: -16.6 cm^2 $volt^{-1}$ sec^{-1}. The changes in the mobilities as a function of the pH have not yet been studied.

In a series of experiments, the fast migrating fractions (component 3, Fig. 9) and the slow fractions (component 1) were drawn from the electrophoresis cell with a syringe and further analyzed. The fast fraction contained large amounts of ribonucleic acid, whereas the slow fraction was practically free from material absorbing light at 260 mμ. Accordingly, chromatography revealed large amounts of ribose in the fast migrating fractions and practically

none in the slow migrating ones. Both fractions contained considerable amounts of glucose, whereas mannose occurred only in the slow fraction.

The eggs as well as the jelly substance are believed to contain polysaccharides or mucoproteins, esterified with sulfuric acid [8, 24]. Since the electrophoretic patterns suggested the presence, in the heated egg extracts, of such types of molecules, a series of sulfate analyses were made. Considerable amounts of acid hydrolysable sulfate were found in all preparations. A

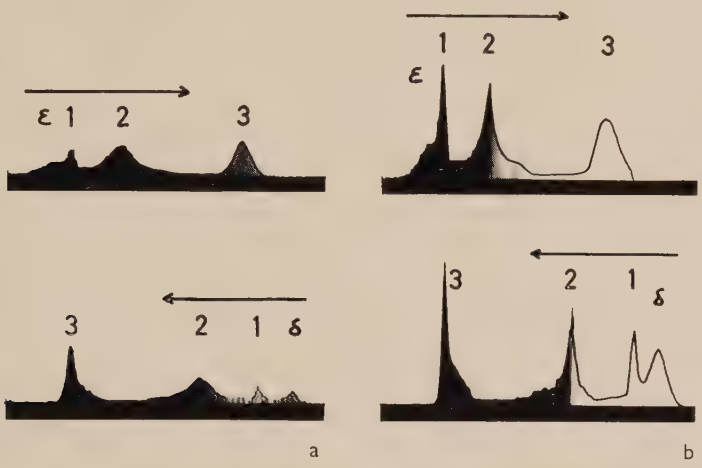

Fig. 9.—Electrophoretic patterns obtained with heated egg extracts, made from frozen eggs (9*a*) or lyophilized eggs (9*b*). Spinco Electrophoresis Apparatus, Model H. Phosphate buffer, pH 7.1, $\mu = 0.1$. Electrophoresis time 100 min. at 12 mA. Upper patterns from descending, lower patterns from ascending boundaries.

rough estimate of the ratio of hexose:sulfate was 4 to 6 for the slow electrophoretic fraction, whereas for the fast migrating, it was 1 or even smaller. These figures are preliminary.

For a further elucidation of the chemical nature of the A-antigen, the egg extracts were subjected to periodate oxidation and mild acid hydrolysis. Details of the treatments are given in the lower part of Table III. The results of a typical inhibition experiment are shown in Fig. 10. As can be seen, the inhibitory activities of the preparations were destroyed (1) by the treatment with periodate and (2) by 4 hours' hydrolysis at pH 1 and 100°C. In contrast to what is the case with the J-activity, the A-activity is retained unchanged after 4 hours' heating to 100°C at pH 3.5. It cannot yet be stated whether the inhibitory capacities of the preparations were correlated with their contents of hexoses. In any case, their inhibitory capacities were not

correlated with their nitrogen content (Table III). They were also unrelated to their content of ribonucleic acid.

As is shown in the first part of this paper, some of the jelly preparations have a considerable A-activity which cannot be correlated with their J-activity. This demonstrates the immunochemical heterogeneity of the isolated jelly substances. This heterogeneity does not become apparent in the electrophoretic analysis;[1] when tested at pH 4 and at pH 8 respectively, all prepara-

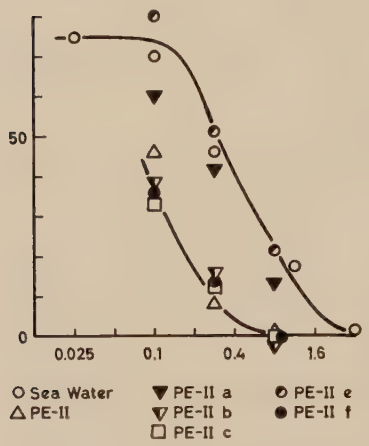

Fig. 10.—500 eggs in 0.2 ml sea water, mixed with aliquots of anti-egg serum P-10, absorbed, or diluted with sea water. *Abscissa*, ml of various antigen solutions used for absorption of one-ml aliquots of antiserum. For explanation of symbols cf. Table III. *Ordinate*, per cent activated eggs. Each symbol represents 200 counted eggs.

tions used in this study gave a single, fast migrating boundary with mobility values of, roughly, $-18\times10^{-5}\,cm^2\,volt^{-1}\,sec^{-1}$. However, the chemical analyses revealed a high degree of heterogeneity; thus, in the preparations described in Table I, the ratios of the fucose to the non-dialyzable nitrogen varied from 7 to 13. Moreover, the fucose:glucose ratios also varied a great deal. This is of particular interest, since the A-specificity is believed to belong to a polysaccharide, and glucose seems to be the only carbohydrate in common both to the egg and to the jelly layer.

In view of these data it seemed reasonable to study the effects of periodate oxidation and acid hydrolysis on the A-activity of the jelly preparations. An experiment of this kind was made with the treated aliquots of the sample PJ-III, described in Table III. The effects of these treatments on the J-inhibiting capacity of the samples have already been shown in Fig. 4. The results of a preliminary experiment with these solutions and anti-egg serum P-11 agreed well with the results shown in Fig. 10. Thus, the heating to

[1] Tested in the micro cell in the Antweiler electrophoresis apparatus, using acetate and phosphate buffers of $\mu = 0.1$. Concentration of substance 0.5 to 1.0 per cent.

100°C at pH 3.5, which partially eliminated the J-activity of the treated preparation, did not affect its A-activity. On the other hand, periodate treatment completely destroyed the A-activity of the sample PJ-IIIa but only half of its J-activity.

DISCUSSION

The results of this study constitute an independent confirmation of earlier findings that the jelly-precipitating and parthenogenetic actions of antiserum on the unfertilized *Paracentrotus* eggs are due to antibodies which are different from each other. The structures which determine the A- and J-specificities of the antigens must, in fact, be greatly different, since cross reactions occur only to a minor degree. Moreover, despite the differences in appearance of the eggs activated by various antisera [16], the same antigen-antibody reaction must be responsible for activation in all cases. Finally, the A- and J-determinants do not represent different antigenic structures of the same molecule but rather belong to different molecules. The A-active molecules occur mainly in the egg, while the J-active molecule is an essential constituent of the egg jelly. This does not exclude the occurrence, in the isolated jelly preparations, of considerable amounts of A-antigen. There are certain indications that the A-activity of a jelly preparation may be lowered if the material is purified with ethanol. This would be in agreement with the differences in the activating potencies of the two anti-jelly sera J-3 and J-4 [16].

The distribution of the A-antigen between the egg and the jelly layer suggests that the A-antigen is peripherically located on the egg and that it may be bound in a somewhat labile manner so that it can diffuse into the jelly. It may also be removed partially when the jelly preparations are isolated. An assumption of this kind would in fact offer an explanation of several phenomena observed earlier; it might explain why the activating action of the antisera on eggs with intact jelly layers is much more variable than on jelly free eggs which, in fact, are easier to activate [15]. Moreover, it might explain why aging of the eggs always reduces their reactivity [16]. In both cases it can be assumed that a considerable amount of the A-antibodies will be bound before they reach the egg surface.

The chemical data presented in this paper are as yet incomplete. However, if considered together with the results obtained in the inhibition experiments after various treatments of different antigen preparations, they suggest that both the J- and A-antigens are of carbohydrate nature. No assumption is made concerning the possible linkages of these antigens in the living egg to

themselves or to other molecules such as proteins or lipids. Moreover, the oxidations with periodate and the hydrolyses were not performed under sufficiently controlled conditions to permit any conclusions concerning a detailed chemical structure of the antigenic determinants. The results of the preliminary experiments described here indicate that it will be possible to analyze these structures in considerable detail.

Since the A-antigen prevails in the denuded egg while the J-antigen is present in the jelly layer, and since glucose is the only detectable carbohydrate common to both the jelly preparations and the heated A-active egg extracts, it might be tempting to correlate the A-activity with a polysaccharide or mucoprotein built up of glucose residues and esterified with sulfuric acid. The occurrence of such substances in the cytoplasmic egg surface of *Paracentrotus* and in those of other sea urchins has been previously demonstrated with the aid of histochemical methods [8, 24]. The existence, in the A-active egg extracts, of a substance containing glucose and sulfate, with an electrophoretic mobility similar to that of the jelly preparations [21, 29, 30] deserves special attention. Moreover, it is of interest that the ratio of fucose to glucose in the different preparations varies considerably. It is hoped that quantitative determinations of the carbohydrates correlated with measurements of the A-inhibiting capacity of various preparations will throw some light on this question.

Despite electrophoretic homogeneity, the jelly preparations differ from each other in some of their chemical and immunological properties. Therefore, there can be no doubt that most of the preparations were heterogeneous. This point is stressed here since the homogeneity of the jelly in the electric field (and in the ultracentrifuge) is sometimes used as an argument in favor of the "fertilizin" theory ([30]; for discussion cf. [16]). Fractional solubility tests, correlated with quantitative tests of the immunological activities, may possibly be of help in further elucidation of these questions.

The data given in this paper are in agreement with the earlier assumption [15, 16] that the activation of the cell is triggered by a particular antigen-antibody reaction. This stimulating action contrasts with the commonly found toxic effects exhibited by antibodies upon addition to cells. Numerous examples of such cytotoxic actions are cited in the recent review of Nace [11]. It should be remembered, however, that the allergic response offers certain analogies [15], since it involves activation of enzyme systems initiated in a direct way by antigen-antibody reactions. In view of the highly specialized functions of the egg surface, the occurrence of an activating antibody among several inhibitory ones is perhaps not surprising. It is well known that

antibodies may act as highly specific enzyme inhibitors [2]. It can also be postulated that the enzyme reactions involved in the onset of egg activation are interlinked in a strict sequence [10, 21, 22]. The interference of an antibody with one of these enzymes may possibly interrupt the state of equilibrium maintained in the cortical regions of the unfertilized egg, and the reactions leading to the activation of the egg may thus be initiated. Moreover, one could assume that the activation may be started by the reaction between an antibody and an enzyme inhibitor (= the A-antigen). In any event, the activating action of the A-antibodies must be rather direct, since a number of other antibodies which also react with antigens in the egg surface do not cause parthenogenesis.

Antigen-antibody like reactions are believed to be of importance in fertilization. Since the reaction of the A-antigen with its antibody may lead to activation, it could well be a receptor substance for the sperm reacting with the sperm head, with the acrosomal filament or with a substance secreted by the sperm. As soon as the chemical nature of the A-antigen is known in greater detail, it should become possible to test any assumption of this kind by means of an inhibition technique, similar to that described in this paper, but applied to the reaction between eggs and sperm instead of to the reaction between eggs and antibodies.

SUMMARY

In the course of an immunochemical investigation of the surface layers of the sea urchin egg, the jelly-precipitating and the parthenogenetic actions of rabbit antiserum on the eggs of *Paracentrotus lividus* were studied by means of antiserum inhibition techniques.

1. The jelly precipitation, obtained with antiserum, is due to the reaction between antibodies and a particular antigen of the egg jelly, the *J-antigen.* The J-antigen is heat stable at pH 7 to 8. It is destroyed when heated to 100°C at pH 1. It is also susceptible to oxidation with periodate and heating to 100°C at pH 3.5. The J-antigen is an integral part of the main polysaccharide of the jelly layer.

2. The parthenogenetic activation of the egg by means of antiserum is due to the reactions between antibodies and a particular antigen, the *A-antigen.* The A-antigen does not belong to the egg proteins destroyed by the action of trypsin and pepsin. It is soluble and heat stable at pH 7 to 8. It is destroyed when heated to 100°C at pH 1 and is also susceptible to periodate.

Thus, the A-antigenic determinants are assumed to have a polysaccharide nature.

3. The A-antigen and the J-antigen are immunologically different molecules. The A-antigen belongs to the egg but it may also occur in the jelly layer. It is assumed that the A-antigen constitutes a part of the cytoplasmic egg surface. There is some evidence which indicates that the A-antigenic principles belong to a molecule, containing glucose and sulfate.

We wish to express our sincere gratitude to Professor John Runnström for his interest and support, and to the staff of the Stazione Zoologica of Naples for the supply of material. We are indebted to Dr. B. Lindqvist at the Central Laboratory of Mjölkcentralen, Stockholm, for valuable help with the analyses in the Spinco electrophoresis apparatus.

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ANALYSIS OF THE SURFACE STRUCTURES OF THE SEA URCHIN EGG BY MEANS OF ANTIBODIES

III. THE C- AND F-ANTIGENS

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In the preceding paper of this series [10] it has been shown that the precipitation of the jelly layer and the parthenogenetic activation which are obtained upon treatment of the unfertilized eggs of the sea urchin *Paracentrotus lividus* with antisera, are caused by the action of distinct antibodies. The antigens involved in the reactions have been called the J- and the A-antigens, respectively. By means of inhibition techniques it has been demonstrated that both are polysaccharides and constitute parts of two different types of molecules.

Jelly precipitation and activation are two of the visible expressions of the action of antisera on eggs. They are only obtained when a small number of eggs are exposed to a relatively large volume of antiserum. No response is obtained if the number of eggs is too high. However, distinct effects can be observed in this case, when the eggs are fertilized after removal of the antiserum [7, 8, 9]. Two phenomena have been described in detail in the first paper of this series, (1) the depression of the rate of fertilization, and (2) characteristic damages to the egg cortex [7]. It was assumed that a number of antigens, probably also located in the surface layers of the eggs but different from the J- and A-antigens, might be involved in these response reactions. The validity of these assumptions has now been studied in a series of inhibition experiments, similar to those described in the preceding paper. The results of these experiments will be presented in the following and the possible physiological significance of the antigens thus far identified will be discussed.

MATERIAL AND METHODS

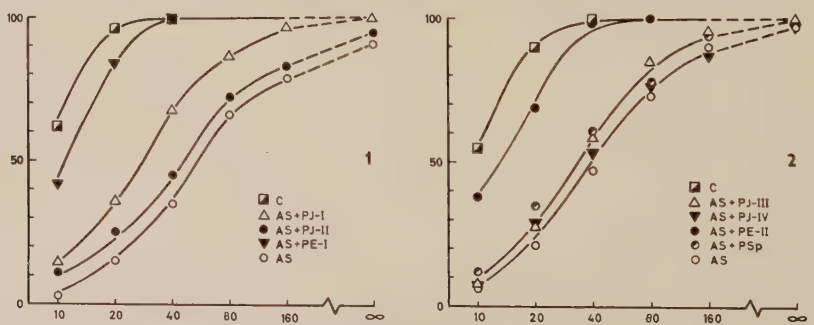
The *antisera* and the *antigen preparations* used for absorption of the antisera were the same as those previously described [7, 10]. Details concerning the *absorption* of the antisera have also been given. Most of the samples of the absorbed antisera employed in this study were taken from batches used in the preceding paper [10].

Inhibition tests.—The experiments were performed with eggs from one female at a time. Only such females were used whose eggs showed complete fertilization and good development in the controls. Aliquots of 0.5 ml sea water, containing ~ 100,000 eggs [7, 9], were added to one-ml mixtures of serum and sea water. For example, 0.8 ml of an *absorbed* antiserum sample, containing 0.4 ml antiserum and 0.4 ml antigen solution in sea water, previously added for absorption, was mixed with 0.1 ml normal serum and 0.1 ml sea water. The effect on the eggs of this mixture was compared with that of (1) 0.4 ml unabsorbed antiserum + 0.1 ml normal serum + 0.5 ml sea water, (2) 0.5 ml normal serum + 0.5 ml sea water, and (3) 0.5 ml normal serum + 0.4 ml antigen solution + 0.1 ml sea water. Thus, the total volumes of the reaction mixtures, the total amount of serum in these mixtures, and the ratio of the number of eggs : amount of antiserum were the same in all cases. The egg/antiserum ratios chosen were the lowest possible, still allowing at least 90 per cent fertilization when unabsorbed antiserum was used. Details concerning duration of treatments, conditions of fertilization, determination of fertilization rate and determination of cortical damage in the fertilized eggs have been described previously [7, 9]. The fertilization rate was defined as the time needed for the fertilization of 50 per cent of the fertilizable eggs in an experiment (= FT 50). The 95 per cent confidence limits served as an estimate of the errors in the FT 50 values. For details cf. [7].

Inhibitory Capacities of Jelly Substance and Egg Homogenate

In the first paper of this series [7] it was found that the two anti-jelly sera J-3 and J-4 had weak fertilization rate-depressing and cortex-damaging potencies, whereas the potencies of the three anti-egg sera P-9, P-10 and P-11 were much higher. It was concluded that the antibodies involved in these response reactions are directed against antigens occurring in the eggs and not in the jelly layers. These conclusions were further tested with the aid of inhibition techniques.

The results of two independent experiments are given in Figs. 1 and 2. The per cent of fertilized eggs obtained after pretreatment with various



Figs. 1 and 2.—Fertilization rate of eggs pretreated with absorbed or unabsorbed antisera. *Abscissae:* Time in seconds of egg–sperm interaction. *Ordinates:* Per cent fertilized eggs. Values based on counts of 200 eggs in each case. C = normal serum; AS = unabsorbed antiserum. For further details cf. Table I.

TABLE I. *Inhibition, with egg-, sperm- or jelly preparations, of fertilization rate-depressing and cortex-damaging actions of anti-egg serum P-10.*

PJ-I, PJ-II, PJ-III and PJ-IV = various jelly preparations; PE-I and PE-II = egg homogenates; PSp = sperm homogenate. For details cf. Tables I and II of the preceding paper [10].

The data are from two independent experiments with the gametes from different sea urchins. The egg/antiserum ratios for pretreatment of the eggs were 2.4×10^5 and 3.2×10^5 for experiments 1 and 2, respectively. The FT 50 values and the 95 per cent confidence limits were calculated from the data plotted in Figs. 1 and 2. All values are given in seconds. Per cent fertilized eggs with cortical damages based on observation of aliquots of 200 eggs 8 10 hours after fertilization.

C = normal serum, c.l. = 95 per cent confidence limits.

Serum	Absorptions: Sample	Absorptions: Amount/ml serum	Fertilization rate: FT 50	Fertilization rate: c.l.	Per cent fertilized eggs with cortical damages
		Experiment 1			
P-10	unabsorbed	—	60.9	54.9–67.5	43.5
P-10	PJ-I	~8000 eggs	27.0	24.5–29.7	32.5
P-10	PJ-II	~5000 eggs	47.4	42.7–52.5	31.5
P-10	PE-I	~6250 eggs	~11.0[b]	—	3.5
C[a]	—	—	~7.0[b]	—	0
		Experiment 2			
P-10	unabsorbed	—	44.8	40.9–49.1	29.5
P-10	PJ-III	~9200 eggs	33.4	30.6–36.4	22.0
P-10	PJ-IV	~7000 eggs	39.8	36.1–43.9	21.5
P-10	PSp	~400 μg N	32.1	28.9–35.6	24.0
P-10	PE-II	~6400 eggs	12.9	11.8–14.1	7.0
C[a]	—	—	9.4	8.3–10.7	0

[a] The results of experiments using normal serum diluted with various antigen solutions did not differ from those obtained with normal serum and sea water, and are not included in the table.

[b] FT 50 values estimated graphically.

absorbed or unabsorbed antisera are plotted as a function of various periods of egg–sperm interaction. Details are given in Table I.

The jelly preparation and egg homogenates used as absorbing antigens were identical with the solutions described in Table I of the preceding paper [10]; the sperm homogenate is described in Table II of the same paper. The egg numbers corresponding to the volumes used for absorption were estimated as described previously [10].

As can be seen from Figs. 1 and 2 and from Table I, the egg homogenates were much more efficient as inhibitors of the depression of the fertilization rate than were the jelly preparations or the sperm homogenate. Table I also shows that absorption with the egg homogenates had a much higher inhibitory action on the cortex-damaging activity of the antiserum than had absorption

with the jelly preparations or with the sperm. Similar results were obtained in experiments with the anti-egg sera P-9 or P-11. The antisera J-3 and J-4 were not tested.

A few specificity controls were also made, employing material from the sea urchins *Arbacia lixula* or *Psammechinus microtuberculatus* for the absorptions. As could be expected, the inhibitory actions of various materials were species specific. This specificity has not yet been studied systematically. Specificity controls were considered less important here than in the study of jelly precipitation and activation, since, in the present study, the antiserum activities were recorded on eggs which no longer were exposed to antiserum when the observations were made. When jelly precipitation or activation is studied, the observations are made in the presence of the *absorbed* antiserum which may contain unspecific inhibitors [10].

The results confirm the previous conclusions [7] that the antibodies responsible for the depression of the rate of fertilization and for the induction of cortical damage are directed against antigens of the egg. From the data it is not clear whether the inhibitory capacities of the jelly preparations are due to cross reactions or to contamination of the preparations with egg antigens. In any event, the J-antigen, which is an integral part of the jelly substance, must be of minor significance in connection with the response reactions considered here.

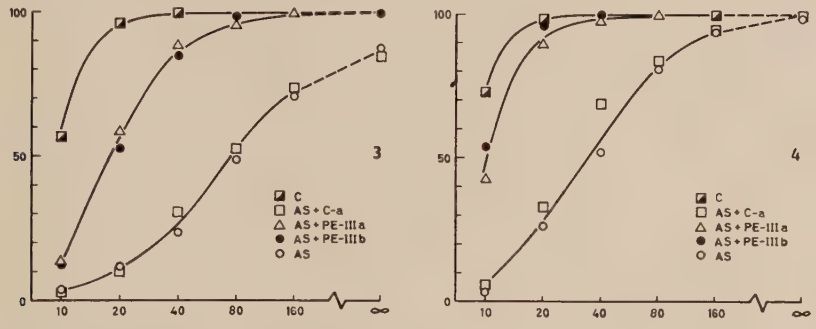
In the preceding paper it was shown that the crude jelly preparation PJ-III exerted a high inhibitory effect on the parthenogenetic activity of the antisera [10]. In the present study this preparation acted as a weak inhibitor, not differing from the other jelly preparations (Fig. 2). Hence, the A-antigen does not seem to be important for the effects described in this paper. This assumption is consistent with the lack of correlation, pointed out previously, between the fertilization rate-depressing and the cortex-damaging potencies of the anti-jelly serum J-4 on the one hand, and its parthenogenetic potency on the other [7].

Some Properties of the Egg Homogenates. The C- and F-antigens

No differences between centrifuged and uncentrifuged egg homogenates can be detected when they are compared with regard to their inhibitory action on the parthenogenetic activity of the antisera. Hence, it was inferred that the A-antigen can readily be dissolved in water [10]. Quite similar observations were made when various antigen preparations were tested with regard to their inhibitory actions on the cortex-damaging activities of the antisera. On the other hand, nothing of this sort can yet be said concerning

the antigens involved in the inhibition of the fertilization rate-depressing activity. In some experiments the *uncentrifuged* egg homogenates were of higher inhibitory capacity than were the soluble parts alone. This might mean that both soluble and insoluble antigens are involved in this response.

In the preceding paper it has been demonstrated that the A-antigen is not susceptible to the action of proteolytic enzymes [10]. A number of experiments with trypsin-treated egg homogenates were also made in the present study. The effects of treatment with pepsin were not studied. The results of two typical experiments are given in Figs. 3 and 4 and in Table II.



Figs. 3 and 4.—Fertilization rate of eggs pretreated with absorbed or unabsorbed antisera. *Abscissae:* Time in seconds of egg–sperm interaction. *Ordinates:* Per cent fertilized eggs. Values based on counts of 200 eggs in each case. C = normal serum; AS = unabsorbed antiserum. For further details cf. Table II.

The egg homogenates used for absorption were identical with the samples PE-IIIa and PE-IIIb, described in Table IV of the preceding paper [10]. The absorbed antiserum samples used here were parts of some of those used in the experiment described in Fig. 6 of that paper [10].

The results show that treatment of the homogenates with trypsin does not reduce their capacity to inhibit the rate-depressing and cortex-damaging effects of the antiserum. Hence, the antigens involved do not belong to that group of soluble egg proteins whose antigenic specificities are destroyed by the action of the enzyme ([10], Fig. 5*a*). The results are similar to those described in the preceding paper [10] with regard to the A-antigen. It should be noted that addition to the non-absorbed antiserum of trypsin and trypsin inhibitor alone had no inhibitory effect, in contrast to what was the case in the preceding study. This is due to the fact that the activating action of antiserum was studied in the presence of these substances, whereas the fertilization rate depression and the cortical damage were studied after these substances had been removed.

TABLE II. *Inhibition, with trypsin-treated or normal egg homogenates, of fertilization rate-depressing and cortex-damaging actions of anti-egg serum P-10.*

PE-IIIa = trypsin-treated homogenate; PE-IIIb = untreated homogenate. Each sample corresponding to ~20,000 eggs/ml. C-a = control containing only trypsin + trypsin inhibitor. For details cf. Table IV of the preceding paper [10].

The data are from two independent experiments with the gametes from different sea urchins. The egg/antiserum ratios for pretreatment of the eggs were 2.2×10^5 and 2.4×10^5 for experiments 1 and 2, respectively. The FT 50 values and the 95 per cent confidence limits were calculated from the data plotted in Figs. 3 and 4. All values are given in seconds. Per cent fertilized eggs with cortical damages based on observation of aliquots of 200 eggs 8–10 hours after fertilization.

C = normal serum, c.l. = 95 per cent confidence limits, d.f. = degrees of freedom.

Serum	Absorptions: Sample	Absorptions: Amount/ml serum	Fertilization rate: FT 50	Fertilization rate: c.l.	Per cent fertilized eggs with cortical damages
		Experiment 1			
P-10	unabsorbed	—	84.1	75.4–93.9	89.5
P-10	C-a	0.2 ml	77.0	69.0–85.9	89.0
P-10	PE-IIIa	0.2 ml	~18.0[b, c]	—	47.5
P-10	PE-IIIb	0.2 ml	20.2	18.8–21.7	51.0
C[a]	—	—	~9.0[b]	—	0
		Experiment 2			
P-10	unabsorbed	—	38.8	35.7–42.2	70.5
P-10	C-a	0.8 ml	~32.0[b, d]	—	61.5
P-10	PE-IIIa	0.8 ml	10.7	9.8–11.7	2.5
P-10	PE-IIIb	0.8 ml	~10.0[b]	—	2.5
C[a]	—	—	~8.0[b]	—	0

[a] The results of experiments using normal serum diluted with various antigen solutions did not differ from those obtained with normal serum and sea water and are not included in the table.

[b] FT 50 values estimated graphically.

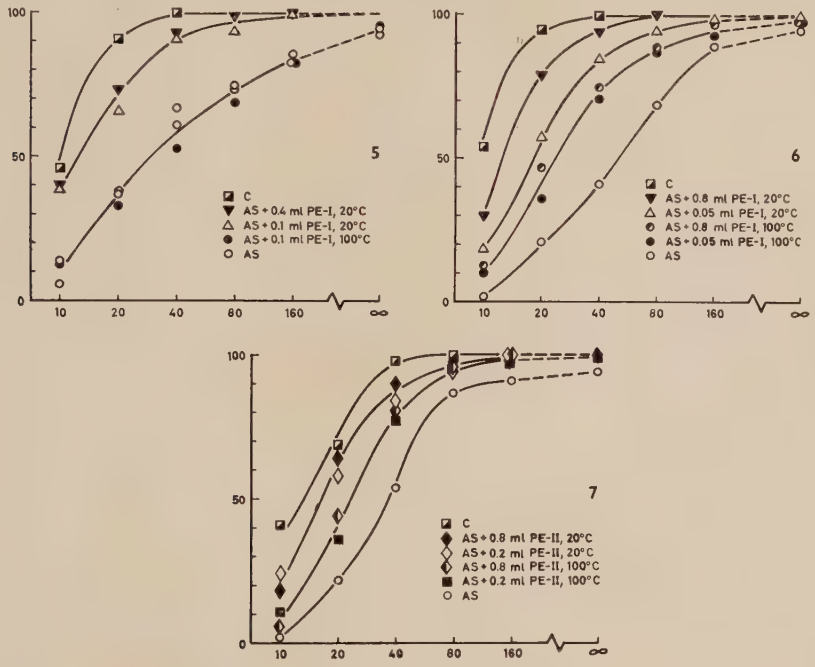
[c] Not linear, $\chi^2 = 8.8$, d.f. = 3, $P < 0.05$.

[d] Not linear, $\chi^2 = 13.3$, d.f. = 3, $P \sim 0.01$.

In view of these findings it was reasonable to study the effect of heating on the inhibiting capacity of the homogenates. The results of three independent experiments are recorded in Figs. 5, 6 and 7 and in Table III.

The egg homogenates used in these experiments were described in Table I of the preceding paper [10]. The absorbed antiserum samples used here were parts of some of those used in the experiment shown in Fig. 8 of that paper.

As can be seen from the last column of Table III, the heating has a striking effect on the inhibitory capacity of the preparations with regard to the cortex-damaging activity; in all three experiments, this capacity is completely destroyed. Hence, the antibodies responsible for the cortex-damaging activity



Figs. 5, 6 and 7.—Fertilization rate of eggs pretreated with absorbed or unabsorbed antisera. *Abscissae:* Time in seconds of egg sperm interaction. *Ordinates:* Per cent fertilized eggs. Values based on counts of 200 eggs in each case. C = normal serum; AS = unabsorbed antiserum. For further details cf. Table III.

of the antiserum must be directed against one or several heat labile antigens, different from the A- and J-antigens described earlier. This is in agreement with the assumptions made in the first paper of this series [7]. The factor may be called the *C-antigen.*

Regarding the fertilization rate depression the results are more difficult to interpret. They are not sufficiently accurate to permit strictly quantitative conclusions. However, there is no doubt that in all cases described heating significantly reduced the inhibitory power of the preparations when compared with that of the unheated controls. Obviously, the C-antigen or some other heat labile factors participate in this response. On the other hand, only in one out of five cases, viz. experiment 1, was the inhibitory capacity of the

TABLE III. *Inhibition, with heated or normal egg homogenates, of fertilization rate-depressing and cortex-damaging actions of anti-egg serum P-10.*

PE-I = Homogenates made from frozen eggs, ~ 125,000/ml; PE-II = homogenate made from lyophilized eggs, ~32,000/ml; 20°C = untreated; 100°C = heated to 100°C for 4 hours at pH 8. All preparations centrifuged prior to absorption. For details cf. [10].

The data are from three independent experiments with the gametes from different sea urchins. The egg/antiserum ratios for pretreatment of the eggs were 3.0×10^5, 2.4×10^5 and 2.1×10^5 for experiments 1, 2 and 3 respectively. The FT 50 values and the 95 per cent confidence limits were calculated from the data plotted in Figs. 5, 6 and 7. All values given in seconds. Per cent fertilized eggs with cortical damages based on observation of aliquots of 200 eggs 8–10 hours after fertilization.

C = normal serum, c.l. = 95 per cent confidence limits, d.f. = degrees of freedom.

Serum	Absorptions: Sample	Absorptions: Amount/ml serum	Fertilization rate: FT 50	Fertilization rate: c.l.	Per cent fertilized eggs with cortical damages
		Experiment 1			
P-10	unabsorbed	—	33.1	29.3–37.3	53.5
P-10	unabsorbed	—	~32.0[b]	—	59.0
P-10	PE-I, 100°C	0.1 ml	40.1	35.6–45.1	64.0
P-10	PE-I, 20°C	0.1 ml	13.6	12.2–15.0	35.0
P-10	PE-I, 20°C	0.4 ml	11.5	10.3–12.9	2.0
C[a]	—	—	~10.0[b]	—	0
		Experiment 2			
P-10	unabsorbed	—	50.0	45.7–54.7	64.0
P-10	PE-I, 100°C	0.05 ml	~27.0[b, c]	—	69.5
P-10	PE-I, 100°C	0.8 ml	24.4	21.8–26.2	65.5
P-10	PE-I, 20°C	0.05 ml	18.5	16.7–20.4	43.0
P-10	PE-I, 20°C	0.8 ml	~8.0[b, d]	—	11.0
C[a]	—	—	~5.0[b]	—	0
		Experiment 3			
P-10	unabsorbed	—	~36.0[b, e]	—	68.5
P-10	PE-II, 100°C	0.2 ml	25.0	23.2–27.0	60.5
P-10	PE-II, 100°C	0.8 ml	23.9	22.3–25.6	58.5
P-10	PE-II, 20°C	0.2 ml	17.7	16.1–19.5	48.5
P-10	PE-II, 20°C	0.8 ml	~15.0[b, f]	—	25.5
C[a]	—	—	~10.0[b]	—	0

[a] The results of experiments using normal serum diluted with various antigen solutions did not differ from those obtained with normal serum and sea water and are not included in the table.

[b] FT 50 values estimated graphically.

[c] Not linear, $\chi^2 = 12.6$, d.f. = 3, $P < 0.01$.

[d] Not linear, $\chi^2 = 6.1$, d.f. = 2, $P < 0.05$.

[e] Not linear, $\chi^2 = 19.0$, d.f. = 3, $P < 0.001$.

[f] Not linear, $\chi^2 = 14.3$, d.f. = 3, $P < 0.01$.

preparations completely destroyed by the heating; in all other cases the FT 50 values obtained with antiserum absorbed with heated homogenates are significantly different from those obtained with unabsorbed antiserum. This indicates that one or several heat stable antigens must also be involved in the rate depression. As pointed out previously in this paper, the A-antigen does not seem to be of importance in this connection. Therefore, it must be assumed that the formation of fertilization rate-depressing antibodies is induced by heat stable antigens, different from the A-antigen (or the J-antigen). These factors, whose molecular identities and chemical characteristics remain to be studied, will be comprised under the name of *F-antigen*.

In the introduction to this section it has been claimed that the inhibitory actions of centrifuged and uncentrifuged homogenates on the fertilization rate depression are often different from each other. The homogenates used in the experiments described here were all centrifuged prior to absorption. This is illustrated when comparing these results with those obtained with uncentrifuged homogenates, shown in Figs. 1 and 2 and in Table I. On the other hand, rather complete inhibition seems to be obtainable with the soluble part alone, provided the amounts employed for absorption are sufficiently large. This suggests that the determinants which absorb antibodies are of the same rather than of different specificities both in the soluble and in the unsoluble parts of the homogenates. This would imply that the heat stable F-antigen is only partially soluble under the conditions used in the preparation of the heated egg extracts. This problem is still under study.

DISCUSSION

The present data indicate that the pronounced damage of cortical breakdown and membrane formation which ensue in the fertilized eggs upon pretreatment with anti-egg serum, is due to the action of antibodies distinctly different from the antibodies reacting with the J- and A-antigens. The C-antigen may belong to the large group of soluble proteins which are denatured by heating of the egg homogenates [10]. This is consistent with the finding that certain antibodies against soluble proteins are absent in the antisera P-11 and J-4 (and J-3); these antisera revealed very low cortex-damaging potencies [7]. On the other hand, the C-antigen is not attacked by trypsin. The results reported in this paper thus give a hint as to how to proceed for the further identification of this factor.

It is assumed that the C-antigen as well as the A-antigen is superficially located on the eggs. In contrast to the A-antigen, the C-antigen must be

rather firmly bound to the eggs, since the isolated jelly preparations have a slight inhibitory effect on the action of the antisera. Furthermore, since the antibodies reacting with the A-antigen and those reacting with the C-antigen seem to interfere very little with each other, we may assume that the two antigens fundamentally differ in their intracellular location and/or in their physiological function. While the A-antigen probably constitutes part of the polysaccharides or mucoproteins of the cytoplasmic surface of the eggs [4, 5, 12], the C-antigen may be part of the cortical granules. In any case, the C-specific molecules must be assumed to play a major role in determining the course of the cortical reaction.

As already stated in the first paper of this series [7], the depression of the fertilization rate represents a more complicated state of affairs. It was postulated that antibodies reacting with several antigens of the egg surface would interfere with fertilization. This has been confirmed by the data given in the present study. Some of the antigens contributing to the depression of the fertilization rate are heat labile. The C-antigen may participate, but this remains to be substantiated. In addition, however, one or several heat stable factors are also involved. The heat stable J-antigen of the main jelly polysaccharide must be of minor importance, as indicated by the weak potency of the anti-jelly sera [7] and the weak inhibitory capacities of the isolated jelly substances. By the same line of evidence the A-antigen can also be ruled out as a factor of importance. Therefore, it must be assumed that the egg extracts contain an additional heat stable fraction participating in the fertilization rate depression, i.e. the F-antigen.

Presumably the F-antigen as well as the A-antigen belong to the layer of polysaccharides or mucoproteins coating the cytoplasmic surface of the egg. It has not been claimed that the A-antigen constitutes the only antigenic molecule of this surface structure. On the contrary, this antigen seems to occur in relatively low quantities, and its extreme solubility certainly represents a rather peculiar feature. The quantitatively predominating part of these structures must be assumed to be more firmly bound to the cell. From the results of histochemical studies it has been concluded that the polysaccharides of the surface are chemically heterogeneous [4]. Moreover, it should be recalled that the heated egg extracts used for antiserum absorption were electrophoretically heterogeneous [10]. There is no reason to believe that they were antigenically homogeneous.

In Table IV the results of this and the preceding papers [7, 10] are summarized. The existence of the four main types of specific molecules, designated with the letters J, A, C and F, seems well established. This does not

TABLE IV. *Various antigens occurring in the surface layer of the eggs of the sea urchin* Paracentrotus lividus.

Antigen	Response to antibody	Localization	Chemical nature of antigenic *determinant*	Possible physiological function of antigenic *molecule*
J	Jelly precipitation	Jelly layer	Heat stable, carbohydrate. Identical with main fucose-sulfate chains in the jelly layer.	Protection of the egg[a]
A	Activation	Belongs to egg cortex, but occurs also in jelly layer (due to diffusion[a])	Heat stable, carbohydrate. — A glucose-sulfate containing molecule[a]	Specific sperm receptor participating in initiation of egg activation[a]
C	Cortical damage. — Fertilization rate depression[a]	Egg cortex	Heat labile. — Protein[a]	Participating in the cortical reactions. — Specific sperm receptor[a]
F	Fertilization rate depression	Egg cortex[a]	Heat stable. Not yet characterized.	Specific sperm receptor participating in sperm attachment[a]

[a] Hypothetical, or conclusions based on indirect evidence.

imply that there exists only one type of molecule, corresponding to each letter and exhibiting the activity in question, nor that additional antigens do not exist in the surface layers of the *Paracentrotus* egg.

The last column of Table IV may deserve some special comments. As can be seen, no particular function was ascribed to the J-antigen, owing to the fact that this antigen was identified by means of an "unphysiological" response reaction, i.e. the jelly precipitation. Thus far, no attempt has been made to correlate the J-specificity with the fertilizin activity [7, 13]. Concerning the possible protective role of the jelly layer cf. references [2, 3, 7, 10, 11].

Judging from the various types of response reactions it may be assumed that the A- and F-factors, and perhaps also the C-factor, participate in the process of fertilization as specific sperm-recepting structures. Thus, they may

facilitate the attachment of the sperm on the egg by means of certain molecular configurations, complementary to configurations on the acrosomal filament and/or on the sperm head. Furthermore, some of the substances may possess prosthetic groups which constitute specific substrates for sperm enzymes. It must be emphasized that no assumption can be made, on the basis of the present studies, as to the identity or non-identity of the antigenic determinants and the various groups within the molecules exhibiting the specific functions in fertilization. Whereas antigen antibody reactions are confined to rather restricted areas on the surface of the molecules, the specificity of the process of sperm attachment is of a much more complex nature. It may involve, in the first stage, a number of more or less reversible steps of mutual attraction. These probably require a certain complementary arrangement of charged groups, which may be extended over relatively wide areas and involve mosaics of several types of molecules. Such steps may then be followed by irreversible steps of a higher degree of specificity, restricted to more limited areas and involving short range forces of attraction. Finally, if activation of the egg is to occur, a spatially oriented union between an enzyme on the sperm and a particular substrate on the receptor sites of the egg may be required.

The response reactions obtained in antiserum-treated eggs of the sea urchin *Paracentrotus lividus* are also obtained with the eggs of several other Mediterranean sea urchins [6, 8]. Hence, the surface of those eggs can be expected to be principally similar to that of the *Paracentrotus* egg. However, considerable differences may occur in the chemical nature of the antigens and in their arrangement in the surface of the eggs from various species.

SUMMARY

The surface structures of the egg of the sea urchin *Paracentrotus lividus* were further analysed by means of antibodies. In this study the cortex-damaging and fertilization rate-depressing actions of antiserum from rabbit on the eggs were studied with the aid of antiserum inhibition techniques.

1. The damage of cortical breakdown and membrane formation, induced in the eggs upon treatment with antiserum prior to fertilization, are due to the reaction between antibodies and a particular antigen of the egg, the *C-antigen*. The C-antigen is different from the J-antigen of the jelly layer and from the A-antigen of the egg cortex. It is soluble in water and is destroyed when heated to 100°C at neutral pH. It is assumed to be a protein although it is not susceptible to the action of trypsin.

2. The depression of the rate of fertilization in eggs pretreated with antiserum is due to the reaction of antibodies with several types of antigens. One or several of these antigens are heat labile (100°C) at neutral pH. In addition, a heat stable factor is involved. This differs from the heat stable J- and A-antigens and is called the *F-antigen*. The F-antigen seems to be less soluble in water than the J-, A- and C-antigens.

3. The localization in the cell and the significance for the process of fertilization of the various antigens are briefly discussed.

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THE FINE STRUCTURE OF SOME CYTOPLASMIC INCLUSIONS OF OXYNTIC CELLS

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Oxyntic (or parietal) cells have been studied by a number of workers. Lim and Ma [6] showed that the granules found in these cells could be stained with Janus green B and thus they presumed them to be mitochondria, and to contain lipid. Menzies [7] showed that the granules contain a phospholipine, and also another lipid, together with a nonlipoidal component. He did not, however, assume the granules to be identifiable with mitochondria, although they clearly have some of their characteristics. In an abstract of some observations on the electron microscopic appearance of parietal cells, Sedar [10] mentioned the presence of numerous mitochondria, which are presumably the objects referred to in the present work as oxyntic granules.

The electron microscopical observations of Palade [8] and others (e.g. [13]) have shown that mitochondria have a characteristic internal structure which appears always to be present, although with slight variations between the cell types. This structure consists of a number of parallel pairs of membranes, the pairs often being nearly parallel to one another. It was thought to be of interest to know if oxyntic granules have a structure similar to this, and it was believed that an electron microscopical study would show whether or not this is the case, and in addition give further information on the cytology of the oxyntic cell.

EXPERIMENTAL

For optimal preservation of cellular detail it is necessary to initiate fixation within a tissue in the shortest possible time following the disturbance of normal conditions. The method found most successful was a perfusion technique, in which the fixative

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is injected into the left ventricle of the heart of a living anaesthetized animal, allowed to perfuse through the entire body, and finally to escape via the severed pulmonary artery. The use of this procedure resulted in good fixation of almost all the tissues.

Twenty to thirty g male mice were used in this study, and the fixative was osmium tetroxide in buffered potassium dichromate [2]. Immediately after perfusion, small pieces of gastric mucosa were dissected out, and placed directly in the mixture for final fixation. The fixed tissue was dehydrated in alcohol and embedded in a mixture of methyl and butyl methacrylate according to standard procedures.

Sections were cut on a Spencer 820 microtome modified for automatic retraction according to Nylén (in the press), adapted for thermal advance [3], and equipped with glass knives. The sections were mounted either on evaporated carbon films or on formvar membranes reinforced with a thin layer of carbon. Examination was carried out in an R.C.A. E.M.U. 2 electron microscope.

OBSERVATIONS

Sections of the region of the gastric mucose which contain the oxyntic cells were difficult to cut owing to the hardness of the granules. Internal structure of a type similar to that found in mitochondria was, however, observed (Figs. 1 and 2). Double membranes, separated by an average distance of 80 Å and of thickness approximately 40 Å were seen, the pairs often being closely parallel with one another.

The granules generally were observed to be denser than mitochondria hitherto observed by electron microscopy, and the double membranes were more closely packed than in most mitochondria. In addition, small dense particles, similar to those observed in the mitochondria of other vertebrate cells, were seen within the oxyntic granules.

Intracellular canaliculi were observed in the cytoplasm of the oxyntic cells, the canaliculi being bordered by many irregular protrusions or microvilli. Sometimes these microvilli gave a similar appearance to the endoplasmic reticulum described by Porter [9]. Multiple membranes within the cytoplasm, having *some* of the morphological features of the inclusion described as Golgi apparatus in other cells (e.g. [5]) have been observed. These will be described elsewhere.

DISCUSSION

It is of interest to find that, in addition to having some of the chemical attributes of mitochondria, the oxyntic granules have also the morphological ultrastructure which has come to be associated almost exclusively with mitochondria. In the abstract of Sedar [10] it is stated that the mitochondria show

an unusually high proportion of closely approximated cristae. Thus, Sedar appears to have no hesitation in calling these granules mitochondria. From the present work it would appear that they are sufficiently close in structure and cytochemical properties to previously observed mitochondria to be classed as such, although they are denser and the internal structure more closely spaced than has been observed so far in the mitochondria of other cells.

Some workers claim to have shown a classical Golgi apparatus in the oxyntic cells [4, 1], while others [11, 12] state that the Golgi element does not exist in these cells. Menzies (personal communication) states that no typical Golgi element is to be seen in any oxyntic cells that he has examined using 'classical' or 'modern' methods and suggests that what Kölster, Beams and King claim to have observed is the lipid envelopes of the oxyntic granules being shed during functional activity, and distorted during fixation. Although inclusions having an appearance similar to that which has been described as Golgi apparatus in previous electron microscopical studies on other secretory cells [5, 14] have been observed in other cells of the gastric mucosa, in the present work structures bearing an unequivocal resemblance to this inclusion have not yet been observed in the oxyntic cell. Sedar, however, describes groupings of tightly packed membranes and associated vesicles resembling the structure described as the Golgi zone by other authors. Sedar further describes a system of vesicles comparable to the endoplasmic reticulum of other cell types, in addition to intracellular canaliculi lined with numerous microvilli or finger-like cytoplasmic projections. The latter observation has been made in the present study, and is evident in Fig. 1. Sections have been observed, however, which have been cut in such a way that the villi or finger-like projections also give an appearance similar to that described as endoplasmic reticulum by Porter [9]. So far, insufficient evidence has been obtained to postulate the existence of endoplasmic reticulum as distinct from the duct convolutions. It is of interest to establish this point one way or the other, and further work is in progress to this end.

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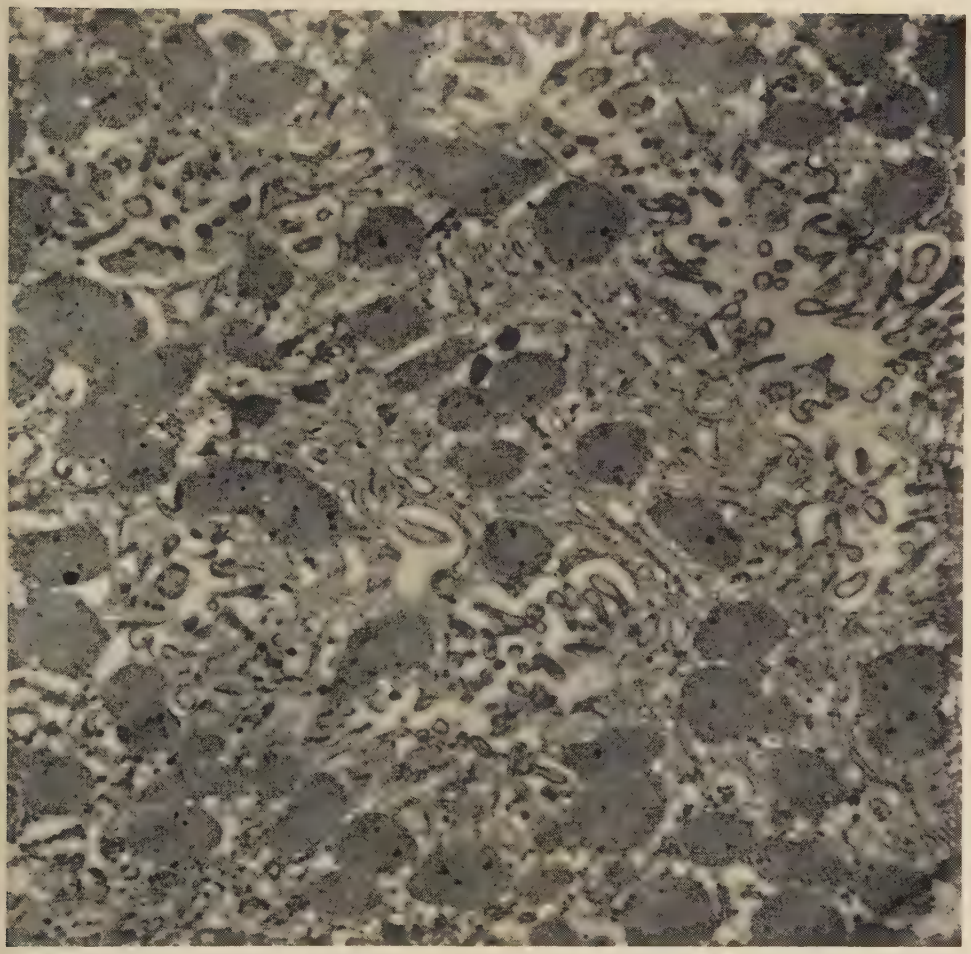

Fig. 1.—Section through the cytoplasm of an oxyntic cell of a mouse showing granules and an intracellular duct, with villar processes protruding from the cytoplasm. × 14,000.

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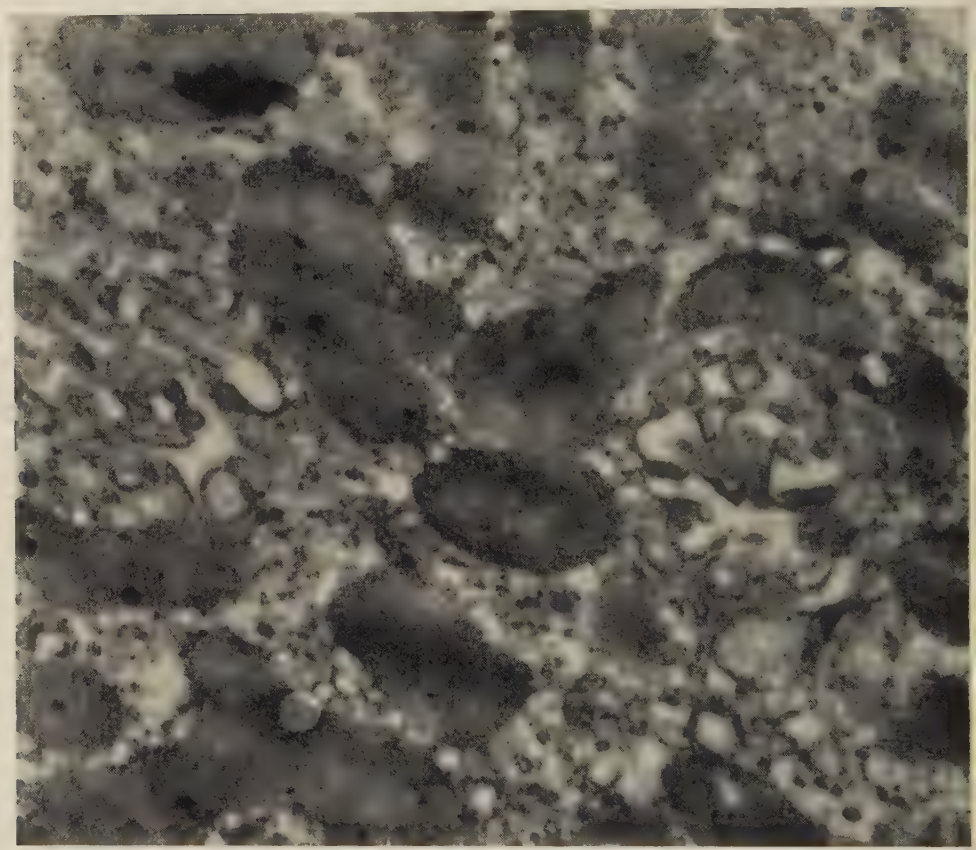

Fig. 2.—Section through oxyntic granules showing lamellar structure similar to that observed in mitochondria and also typical small dense particles within the granules. × 37,000.

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CINÉ-MICROGRAPHIC STUDIES ON MITOSIS IN ENDOSPERM

III. THE ORIGIN OF THE MITOTIC SPINDLE[1]

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In plant cells, whether living or fixed, it is usually difficult to distinguish exactly the border between the spindle and the outer cytoplasm. This may help to explain why information about spindle development is so scarce in the literature.

It has been possible to record by cinematography the development *in vitro* of the spindle during mitosis in many endosperm cells of various species, since in this material the process is clearly visible.

MATERIAL AND METHODS

The endosperm of *Colchicum autumnale*, *Iris aphylla*, *Clivia cytranthiflora*, *Leucojum aestivum* and *Haemanthus Katharinae* was investigated. The medium consisted of 0.4–0.5 per cent agar with glucose; the concentration of glucose used depended on the species, being 3 per cent for *Colchicum*, 3.5–4 per cent for *Iris*, 3 per cent for *Leucojum*, 3.5 per cent for *Clivia* and 3.5 per cent for *Haemanthus*. Some, but not all cells were flattened experimentally; this flattening resulted from surface tension when, in preparing endosperm fluid, a small volume was thinly spread. The methods of handling endosperm *in vitro* are described elsewhere [2, 5] as are also the methods of film analysis [6] which, however, have been somewhat modified for the present investigation. In the analysis of the film record two copies were always used, one for projection and the other for simultaneous measurements. For noting the exact beginning and the end of some events, the copy was marked with a grease pencil, the marks being discernible during projection. The timing of a number of events is facilitated by placing marks in various places, the marks in any one posi-

[1] This paper, as well as Nos. IV and V of the series are concerned mainly with the analysis of the film *Mitosis in Endosperm*, made by A. Bajer and J. Molé-Bajer (Film Polski, Warsaw, 1955). Sequences from this film are here referred to by the numbers printed in the film itself. The author is especially grateful to Dr. J. Boss and Dr. H. B. Fell of the Strangeways Research Laboratory, Cambridge, England, for most valuable suggestions and much help during the preparation of the manuscripts of this series (III V). The author would like to express his gratitude to Professor T. Teleżyński of Warsaw University and Dr. G. Östergren of Lund University for valuable criticism and discussion of several problems.

tion corresponding to a particular event. This method is quick, convenient and very exact, the error not exceeding ½–1 minute.

A Zeiss phase contrast microscope with 90× oil immersion lens and 4× eyepiece was used throughout. Photographs were taken on Agfa "Superpan" 35 mm film at the following rates: 1.5–12 frames per minute for *Haemanthus*, 6–8 frames/min. for *Clivia*, 6–10 frames/min. for *Colchicum*, 8–12 frames/min. for *Iris* and 5 frames/min. for *Leucojum*, projection being always at 24 frames per second.

OBSERVATIONS

The formation of the clear zone.—The first sign of the spindle is the appearance of a clear zone (in [1, 6] called clear space) around the nucleus (Fig. 3, first photograph). Though this process has been briefly described in the previous papers of this series [1, 6], it was not analysed in detail.

The formation of the clear zone is most clearly discernible around the large nuclei of *Haemanthus*; the diameter of these nuclei, when they are rounded, is about 30 μ, and attains 50–60 μ after flattening, the nuclear

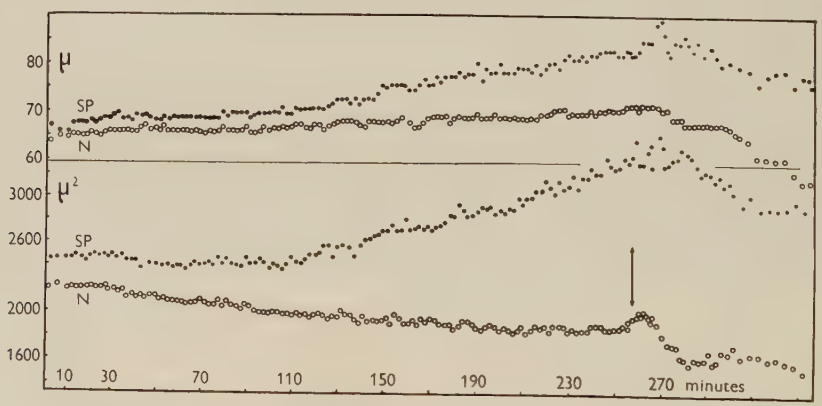

Fig. 1.—*Spindle formation.* In the upper part of the graph the diameters of the nucleus and spindle are plotted against time. *SP* is the diameter of the outer limit of the clear zone measured in the direction of the future long axis of the spindle, and later of the spindle itself similarly measured. *N* represents the diameter, first of the nucleus, and then, after the dissolution of the nuclear membrane, of the chromosome group. The difference between *SP* and *N* therefore is a measure of the width of the clear zone where it is cut by the diameter along which measurements were made. The lower part of the graph is similar to the upper, but represents changes in area, not diameter: thus the difference between these is the area of the clear zone or the spindle, according to the stage of development. The clear zone begins to enlarge markedly at the 110th minute, but the nuclear area decrease before this, and continues to do so afterwards. The arrow indicates the time of onset of the contraction stage. Immediately after dissolution of the nuclear membrane, the chromosomes occupy a larger area, but this, like the reduction in area which occurs in the contraction stage, is more striking in the projected film than in the graphs. This is a half-flattened cell; in a strongly flattened cell (Fig. 2) the processes are more clearly visible. *Haemanthus Katharinae.* Cell No. 421. Film sequence No. 40. Micrographs in Fig. 3.

volume being over 10,000 μ^3. The formation of the spindle is illustrated in Figs. 1–3, with which may be compared Fig. 1 of paper IV in this series [3]. In *Haemantus* the clear zone first appears in mid-prophase, some hours before the disappearance of the nuclear membrane. For a long time after

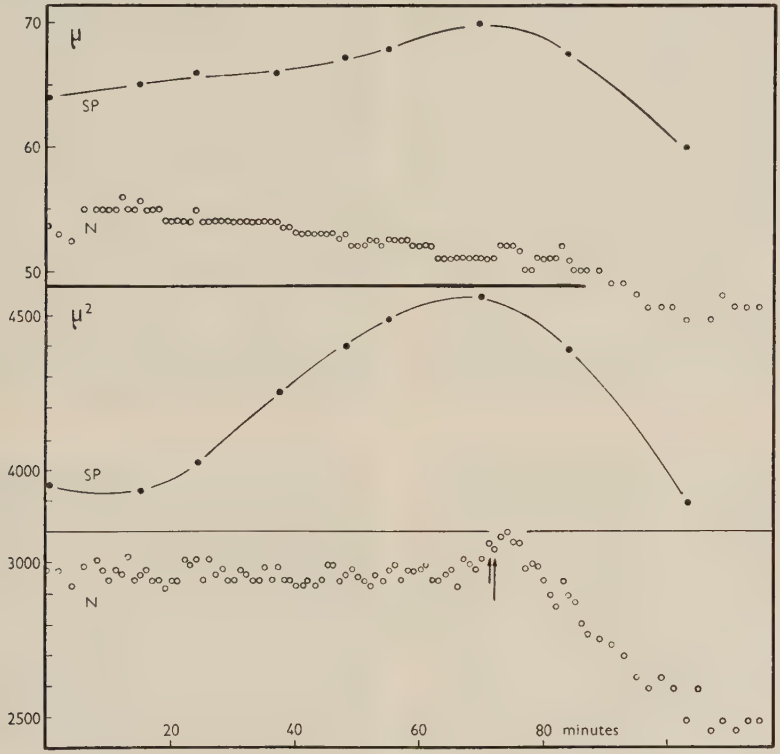

Fig. 2.—*Spindle formation.* Two pairs of graphs comparable with Fig. 1 in all respects, save that the cell used for the measurements in this figure is extremely flattened. The two arrows indicate the onset of the contraction stage, the short arrow being placed before the dissolution of the nuclear membrane and the long one after it. The difference between *SP* and *N* in the lower part of the figure represents the area of the clear space, and later of the spindle; this area can be seen to increase rapidly, while the nuclear area remains unchanged. The decrease in the area occupied by chromosomes and spindle after the beginning of the contraction stage is very clear. *Haemanthus Katharinae.* Cell No. 405. Film sequence No. 26. (Photomicrographs of this cell have appeared in [5], Fig. 4.)

this stage the zone enlarges extremely slowly or not at all, and the variations in its dimensions during this period are usually within the limits of experimental error; in this respect, direct observations during projection agree with graphs derived from the film record. For some time after its first appearance (1 ½–4 hours in *Haemanthus*) the clear zone extends only 2 ½–5 μ

beyond the nucleus, but it then begins to enlarge rapidly. During film projection the area of the clear zone is seen to expand in all directions perpendicular to the nuclear surface. This expansion continues for about 30–60 minutes in rounded cells and for 1–2 hours in flattened ones, until the clear zone reaches a diameter of up to 80 μ. The expansion is usually equal in all directions in flattened and isodiametrical cells, but in most elongated cells the clear zone is correspondingly elongated so as to resemble the structures described as polar caps [11, 15]. According to Martens [11] whose opinion is shared by Telczyński [15], during prophase, in the places where the spindle poles later develop, clear or fibrous, often semilunar, caps are formed; they are called polar caps. Some authors have maintained, however, that polar caps form within the nuclear membrane and not outside it, as does the clear zone. While the clear zone is expanding, its outer border is usually not sharp and distinct, although in extreme examples it may be either very sharp or entirely absent [3]. The area of the nucleus in optical cross-section and sometimes also its volume diminish (cf. p. 500) during expansion of the clear zone; this fact is important in explaining some of the factors responsible for its origin.

The velocity of the radial spread of the clear zone varies, it does not exceed 0.2 μ/min. in *Haemanthus*. The graph may be a straight line (Fig. 1 of this paper; Fig. 1 [3]; film sequence 36—*Colchicum*), but usually the velocity first increases and then decreases (Fig. 2; Fig. 3 [3]).

The clear zone forms in close relation to the chromosomes, and when protrusions of the nucleus extend into corresponding cytoplasmic pockets during prophase (see below), they are always surrounded by the clear zone. As prophase proceeds, these nuclear protrusions may be wholly or partially retracted. Usually a protrusion contains only one chromosome limb, which is nevertheless sufficient for the formation of an adjacent clear space.

These nuclear protrusions are not exceptional, and should not be regarded as abnormal. They are formed as a result of a previous anaphase in which the limbs of some chromosomes, usually only a few, precede the kinetochores. In late anaphase these limbs are poked into the cytoplasm at the spindle poles. Such behavior of chromosome limbs in anaphase is fairly common, and has been observed by several authors [9, 14]. The protruding limbs are often retracted into the nucleus in telophase, and protuberances of interphase nuclei are therefore rare.

As the cytoplasm is transformed into the clear zone, the first structural change is the elimination of the tiny particles which make up its granular contents.

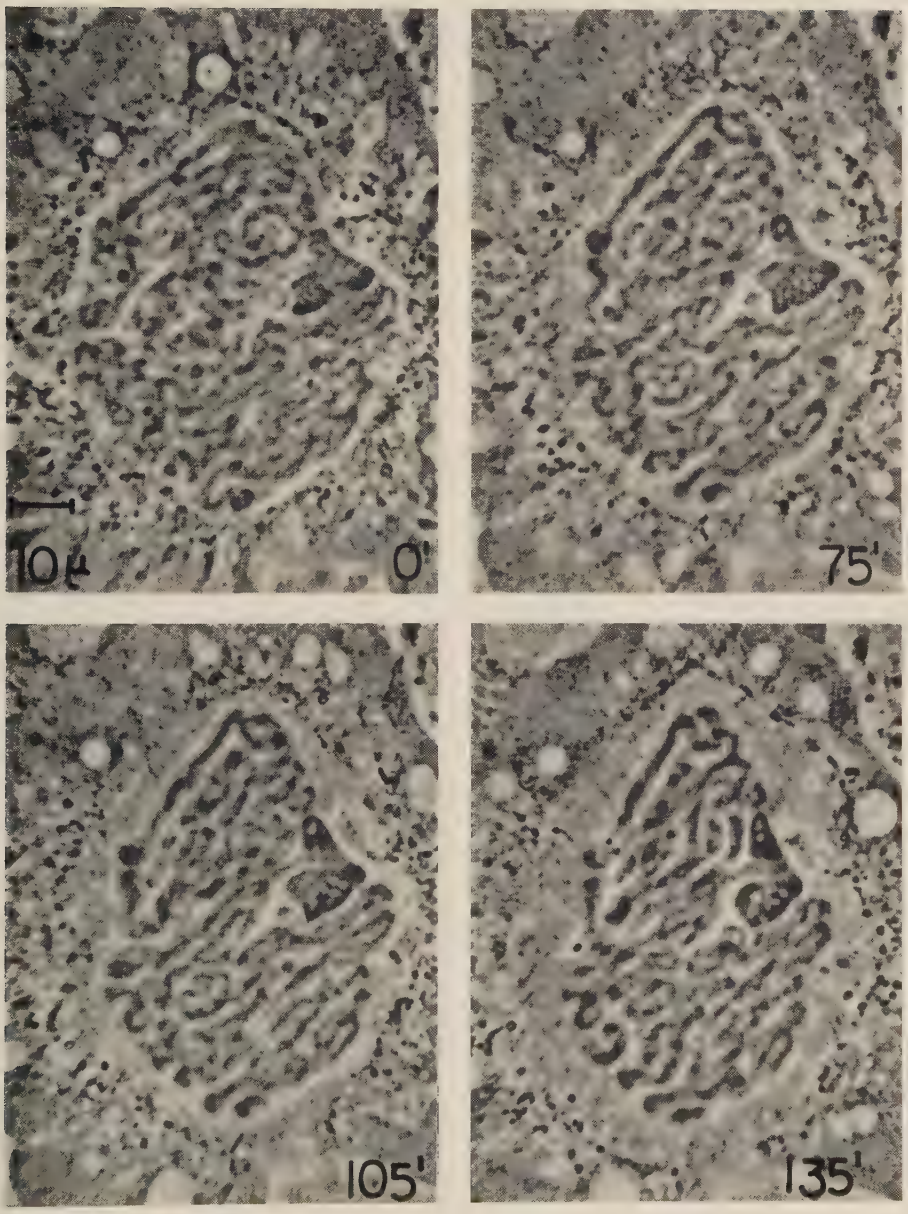

Fig. 3.—*Growth of clear zone and spindle formation in Haemanthus Katharinae.* Living cell. Time in minutes indicated on micrographs. The contraction stage is seen at 215 minutes. Fig. 1 shows graphs derived from this cell. Phase contrast. Scale on first photograph. Cell No. 421. Film sequence No. 40.

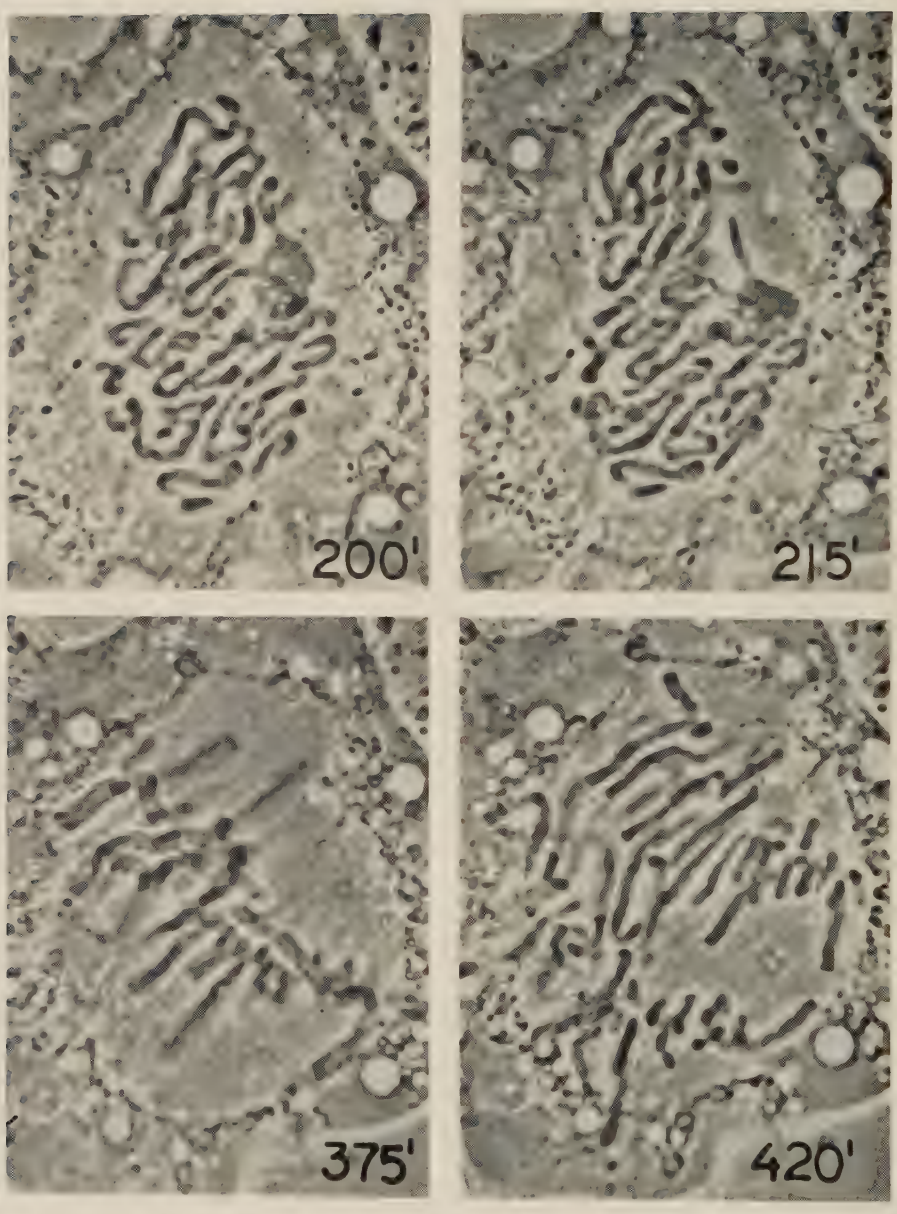

Fig. 3, continued.

That the structure of the clear zone is unoriented is indicated (1) by the absence of any clearly defined long axis for the incipient spindle, and (2) by the presence of Brownian movement in the area.

(1) In cells with cellulose cell walls, the orientation of the spindle axis depends on mechanical and other conditions and this matter has been much discussed [8]. In endosperm cells, which have no cell walls, the conditions are different; for in these, the long axis of the spindle is often, but not always, parallel to that of the cell. Often, in elongated cells, the clear zone is not of equal width in all directions but stretches toward the cell poles, so that the development of the clear zone may determine the polarity of the spindle, which is then the same as that of the cell. In cells in which the clear zone is developed to an even width, as it usually is in flattened and isodiametrical cells, it is impossible at this stage to predict the direction of the long axis of the future spindle, which is quite independent of the shape of the nucleus. It seems probable that the axial orientation of the spindle depends partly on an intranuclear kinetochore arrangement and on mechanical conditions during the contraction stage, especially the direction of apparent compression at this stage. The factors responsible for the final orientation of the spindle axis in endosperm are at present unknown.

(2) Tiny particles in Brownian movement (cf. [3]), are very rare in the clear zone, having been almost entirely eliminated during its formation. The few remaining particles oscillate irregularly in all directions, although there is a particular tendency to oscillate perpendicularly to the nuclear surface.

At this stage the clear zone is semi-liquid and it may flow out from the cell when, for example, flattening has been too abrupt. When this outflow was watched in cells of *Clivia* and *Haemanthus* it was seen that the time taken for metaphase plate formation was not prolonged.

In general, the formation of the clear zone proceeds in this way in all the material studied, but in each plant species there are special features correlated with the size of the chromosomes. Thus, in *Haemanthus*, in which the chromosomes are very large, their limbs are frequently found in nuclear protrusions; on the other hand the smaller chromosomes of *Leucojum*, for example, seldom behave in this way, while in other species with yet smaller chromosomes, protuberances containing chromosome limbs are absent. Although, in *Leucojum*, the clear zone usually develops only weakly, it is often very well marked in *Haemanthus*. It is also obvious that precise details are more easily seen in cells with large chromosomes than in those with small ones.

In cases where the clear zone is formed most rapidly, there are very vigorous cytoplasmic movements; these are not directed currents but give the impression of a bubbling which is most vigorous when the formation of the clear zone is proceeding at its fastest rate.

Nuclear changes.—The question arises whether the formation of the clear zone may not be correlated with a change in nuclear volume and whether it may not largely result from the migration of material through the nuclear membrane. In order to study the nuclear volume exact measurements were made.

During the period immediately before the dissolution of the nuclear membrane, all nuclei of endosperm cells tend to become spherical or ellipsoidal. The shortening of chromosomes sometimes leads to the withdrawal of nuclear protrusions, while the outline of the nucleus becomes increasingly oval. Just before the nuclear membrane disappears the chromosomes are evenly distributed throughout the nucleus. Some cells clearly undergo greater changes in shape than others, and large cells in particular alter the most. To investigate the changes in nuclear volume, the cross-sectional area was measured in flattened and unflattened cells (Figs. 1, 2 and Figs. 1, 3 in paper IV). This cross section is presumably the optical section, i.e. the apparent nuclear area. It was found that the cross-sectional area increased only in those cells which became flatter as the clear zone formed. Conversely, those cells which did not flatten or did so only slightly, showed a decrease in the cross sectional area of the nucleus, which may have been due to a rounding of the nucleus, to a change in its volume, or to both processes combined. It must be stressed, however, that changes in nuclear volume can be inferred from measurements on film records for very flattened cells only, otherwise a simultaneous change of depth may mask an alteration in volume. The changes in Fig. 1 are not as clear as in Fig. 2 because the cell in Fig. 1 is less flattened than that in Fig. 2. Alterations in nuclear volume are best determined in extremely flattened cells which show no tendency to flatten further (Fig. 2). In *Haemanthus* cells of this kind the nuclear cross-sectional area is about 2500–3000 μ^2 and the thickness may be considered as constant at 5–8 μ, the exact figure depending on the individual cell. In these flat cells the change in area would be proportional to the change in volume. As no great change in volume was noted in any very flattened cell, it may be concluded that, from the appearance of the clear zone (first sign of spindle formation) until the contraction stage, the nucleus decreases slightly in volume or, in rare examples, does not change. The changes in nuclear volume are slight and cannot, volume for volume, account for the growth of the

clear zone. Despite this absence of a quantitative relationship, a qualitative one is very probable.

In the preceding account the clear zone has been considered as a cytoplasmic constituent of the mitotic spindle, though small amounts of nuclear substance may be present in it. During the contraction stage the clear zone acquires nuclear components by mixture with the nuclear sap, and the clear zone and the sap, by interaction with the kinetochores, form the spindle, within which the chromosomes then are moving. The contraction stage which follows the dissolution of the nuclear membrane is a peculiar phase in which the chromosome group is compressed. A detailed analysis is given elsewhere [3].

DISCUSSION

The present observations indicate that, in accordance with Mazia's views [12, 13], the spindle is, at the outset, of cytoplasmic origin. The chemical changes inside the clear zone were not studied, but it seems highly probable that the zone forms in response to substances which pass through the nuclear membrane from the nucleus into the cytoplasm. It is difficult to draw any conclusion as to what type of substance could transform cytoplasm into the clear zone, a change which is characterised morphologically mainly by the elimination of visible particles from the area of the future spindle. The results fail to confirm the view that large quantities of material come out of the nucleus at the beginning of spindle formation. Some earlier authors were of this opinion. Jungers [10] described a "substance périnucléaire" which is nothing else than the clear zone; he maintains that this substance is of nuclear origin. Becker [7] who, on the basis of vital staining experiments, maintains that the whole mitotic spindle is of nuclear origin. Becker's view is shared by Wada [16]. Becker and Wada studied staminal hairs of *Tradescantia*, material which is not suitable for detailed observations of this kind. This may explain why the results are different from those described here. In endosperm, on the other hand, it was found that when the clear zone is enlarging most rapidly, the nuclear volume changes very little or not at all. The formation of polar caps may be explained as the tendency of the clear zone to assume a polar distribution in the more elongated cells, although, in the fixed material used by some previous workers, the appearance of polar caps may be due to the configuration of the incipient spindle during the contraction stage. *In general, polar caps may be a special form of the clear zone.*

Once the clear zone has formed, the further development of the spindle depends principally on the dissolution of the nuclear membrane.

SUMMARY

1. The clear zone, which is the cytoplasmic contribution to the mitotic spindle, forms around the nucleus, appearing in early prophase and rapidly enlarging in late prophase.

2. In *Haemanthus Katharinae* the slow growth of the zone may last for over 4 hours, while subsequent, more rapid development occupies a further 30 min. to 2 hours. During all this time the decrease volume of the nucleus is but slight.

3. The clear zone has no definite structure and, unlike the fully formed mitotic spindle, it is semi-liquid and may flow out from the cell.

4. The quantity of the substance which may migrate from the nucleus into the clear zone is very small in relation to the growth of the clear zone and its quantitative influence may be neglected.

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Experimental Cell Research **13**, *503–509 (1957)*

INHIBITION OF CELL DIVISION BY ULTRAVIOLET IRRADIATED UNSATURATED FATTY ACID[1]

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The action spectra for retardation of cell division by ultraviolet light suggests that nucleoproteins and proteins may be sites of action [5]. Unsaturated lipids are also present in cells. The ultraviolet irradiation of unsaturated fatty acids *in vitro* is known to produce peroxides which in turn affect cell constituents. Some of these effects are as follows. Crude deoxyribonucleoprotein preparations from rat spleen are depolymerized by lipid peroxides but not by the corresponding unoxidized fatty acid esters [3]. Irradiation of rat liver mitochondria produces lipid peroxides, and the inhibition of certain oxidative enzymes which results is proportional to the amount of peroxide formed [7]. Finally, the growth of *E. coli* is inhibited by lipid peroxides [8]. Latarjet gives evidence for the radiomimetic action of other organic peroxides [6]. It seemed possible therefore that lipid peroxides might account for some of the effects of ultraviolet light.

If lipid peroxides are concerned in ultraviolet effects, then physiologically effective doses of irradiation should cause lipid peroxide formation in intact cells. Further, irradiated lipids brought into contact with dividing cells may produce some of the cytological and inhibitory effects of ultraviolet light. We have examined these points using the fertilized eggs of the annelid *Chaetopterus pergamentaceous* and the sea urchin *Lytechinus variegatus*.

METHODS

Chaetopterus eggs washed free of body fluids and contained in 2 ml sea water were irradiated in dishes 5 cm in diameter placed in a constant temperature bath at 29°C. Dishes of experimental eggs were covered with a quartz plate and control eggs were covered with glass. A GE H-4 ultraviolet lamp without filter was used for the irradiation. Irradiation was begun approximately 5 minutes after fertilization.

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[2] Operated by the Union Carbide Nuclear Company for the United States Atomic Energy Commission.

Lipid peroxides resulting from irradiation were measured by the thiobarbituric acid (TBA) colorimetric method [9]. Irradiated eggs in 2 ml sea water were placed in trichloroacetic acid (1 ml 32 per cent) and TBA (2 ml 0.67 per cent) and heated in boiling water for 10 minutes. The optical density of the resulting color in the supernatant was measured at 535 mμ in a Beckman DU spectrophotometer.

For the study of the effects of methyl linolenate on dividing eggs, sea water extracts of methyl linolenate (Hormel), both in the unoxidized state and after oxidation by ultraviolet irradiation, were prepared by shaking 0.5 ml of linolenate with 10 ml of sea water in a separatory funnel for 5 minutes. The emulsion was allowed to stand for one hour after which the sea water was withdrawn from the separated ester. The degree of linolenate oxidation was measured by the TBA reaction. The extracts were added to the eggs 5 minutes after fertilization.

Cleavage time was determined for first, second and third cleavages by rapid counts of 100 eggs or by fixing samples with formalin at intervals during cleavage. In both methods the time of 50 per cent cleavage was determined by interpolation. It was found that oxidized methyl linolenate brought about reversal of furrow formation and blockage rather than simply delay in cleavage. In view of these results the percentage of eggs reaching third cleavage at the time of cleavage in the control eggs was used as a criterion of effect.

For the cytological studies, *Chaetopterus* eggs were fixed in Bouin fixative, sectioned at 10–15 μ, and stained with Feulgen and hematoxylin. *Lytechinus* eggs were fixed in acetocarmine and mounted in a Karo syrup—aceto-carmine—water mixture 2:3:3. (Karo syrup is a heavy sugar syrup.) The transparency of the latter eggs permitted observations without the necessity of sectioning.

RESULTS

The irradiation of fertilized eggs of *Chaetopterus* resulted in an increase in lipid peroxides (Fig. 1) shown by the increased absorption at 535 mμ. (The absorption peak at 460 mμ is characteristic of carbohydrates in this reaction.) The amount of peroxides formed was proportional to the period of exposure (Fig. 2). The curves at the right show two experiments. With a 30-minute exposure, a clear-cut increase in lipid oxidation always occurred. After a 10-minute exposure an increase was observed in some cases but not in others.

In another series of experiments fertilized eggs were irradiated under conditions precisely similar to those used for the lipid peroxide studies in order to determine the degree of cleavage inhibition with different periods of ultraviolet irradiation. The results of one of the experiments are plotted in Fig. 2 in the curve at the left. Following an exposure of 20 minutes, only 24 per cent of the eggs completed third cleavage; and with 25 minutes exposure, the third cleavage was completely suppressed. From a comparison of cleavage inhibition and lipid peroxide formation shown in Fig. 2, it can

be seen that peroxide formation occurred at doses of ultraviolet light which inhibited cleavage.

The next step was to ascertain the action of irradiated fatty acid on dividing cells. It was found that aqueous extracts of irradiated methyl linolenate were strongly inhibitory, whereas extracts of the unirradiated ester were without effect on cleavage (Table I). The inhibitory material in 2.5 ml of extract was equivalent to about 3 μg of completely oxidized linolenate.

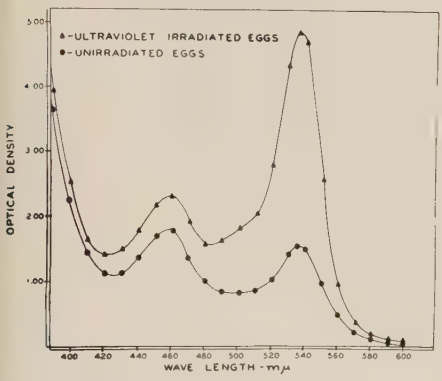


Fig. 1.

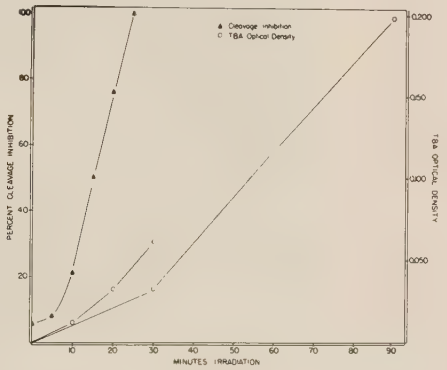


Fig. 2.

Fig. 1.—Absorption spectra of TBA color reaction of *Chaetopterus* eggs.
Fig. 2.—Effect of UV on lipid peroxide formation and cleavage inhibition of *Chaetopterus* eggs. Lipid peroxide formation (right ordinate) in two experiments shown in curves at right. Cleavage inhibition (left ordinate) shown in curve at left. Conditions constant for lipid peroxide and cleavage experiments.

The inhibition of cleavage by irradiated linolenate was due in part to the reversal of furrow formation. This was demonstrated by determinations at short intervals of the percentage of eggs with a clear-cut cleavage furrow. Table II presents the results from four experiments on the maximum and final percentage of eggs showing cleavage furrows. The final percentage (from a series of estimates on the same batch of cleaving eggs) in the last column is seen to be lower in all cases, indicating a reversal of furrow formation by some of the irradiated eggs. However, furrow reversal does not completely account for the observed inhibition since in some eggs furrow formation was never initiated. In addition abnormal shape changes occurred after treatment with irradiated linolenate extract and after extended exposure of the eggs to ultraviolet light.

Mitotic abnormalities following treatment with irradiated linolenate have been observed in stained sections of *Chaetopterus* and in the living trans-

TABLE I. *Cleavage inhibition by ultraviolet irradiated methyl linolenate.*

Sea water extracts of the fatty acid ester were added to fertilized eggs 5 minutes after fertilization. The TBA value of the irradiated ester extract was 21.7 optical density units/ml, while that of the unirradiated ester extract was 2.9 optical density units/ml.

ml linolenate extract per 10 ml sea water	% cleavage inhibition
Irradiated linolenate:	
0	1
1.5	10
2.0	37
2.5	99
Unirradiated linolenate:	
2.0	1
2.5	0

TABLE II. *Reversal of furrow formation by ultraviolet irradiated methyl linolenate.*

The percentage of eggs showing furrows was determined at frequent intervals during cleavage. The table presents only the maximum and final values from a gradually declining series.

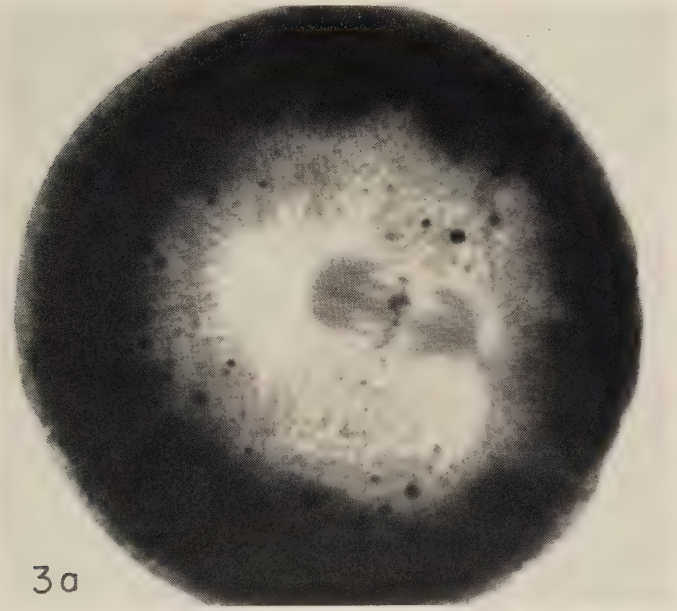
Experiment	Maximum % of apparent cleavage	Final % of cleavage
1	65	37
2	87	77
3	46	28
4	36[a]	8

[a] 82 per cent showed incipient furrow formation.

parent eggs of *Lytechinus*. Inhibition of aster and spindle formation occurred when eggs were exposed to irradiated linolenate extract prior to metaphase (Fig. 3). Fully formed asters and spindles of *Lytechinus* were also reduced in size when treated at the time of metaphase.

Exposure of *Chaetopterus* eggs to ultraviolet light in doses just sufficient to block cleavage completely resulted in reduced asters and spindles similar to those obtained with exposure to the irradiated linolenate. The spindles observed in stained sections of these irradiated eggs were extremely reduced in

Fig. 3.—*Chaetopterus* egg spindles seen in stained sections. (*a*) normal egg at metaphase; (*b*) egg treated with linolenate peroxide showing much reduced metaphase spindle.



3a

3b

size in over ninety per cent of the spindles counted in two separate experiments. Although the apparent proportion of reduced spindles would tend to be increased by incomplete staining, this proportion might also be decreased by extensive radiation damage which could eliminate the spindle entirely.

DISCUSSION

Both ultraviolet light and lipid peroxides were found to inhibit cleavage, to reduce spindle size, and to cause abnormal shape changes in fertilized eggs. Lipid peroxides are formed in these cells as a result of ultraviolet irradiation. It is therefore reasonable to assume that some of the effects of ultraviolet light are mediated by these organic peroxides. Hydrogen peroxide, on the other hand, as shown by Giese *et al.* [4], does not produce effects similar to those of ultraviolet light. It is possible that lipid oxidation products other than peroxides may also play a role. Surface activity of the added lipid peroxide probably is not concerned since the methyl ester of linolenic acid, which is non-polar, was used.

Lipid peroxides undoubtedly act directly on fibrous proteins of the cell as shown by their action in reducing the viscosity of deoxyribonucleoprotein gel of rat spleen [3] and thymus [2]. This need not be their only action, however, for these compounds have been shown to inhibit certain oxidizing enzymes [7] and so could interfere with synthetic activities of the dividing cell. The small size of the spindle might be explained in this manner. But the fact that an extract of oxidized linolenate caused a reduction in size of the fully formed spindle of *Lytechinus* demonstrates a more direct action. Carlson and Hollaender have demonstrated a reduction in spindle size with ultraviolet radiation applied to the fully formed spindle [1]. The inhibition of cleavage, the reversal of furrow formation, and the irregular shape changes may be due to disruption of the cortical gel structure. This disruption may result directly from the action of the added oxidized linolenate, or in the case of ultraviolet damage, through the agency of lipid peroxide formed as a result of irradiation. The plasma membrane structure of the egg may also be weakened. In either event, protein could be the site of action.

SUMMARY

Ultraviolet irradiation of eggs of *Chaetopterus pergamentaceous* resulted in the formation of lipid peroxides as measured by the thiobarbituric color reaction.

Aqueous extracts of ultraviolet irradiated methyl linolenate inhibited cleavage, reversed furrow formation, and reduced spindle size. These effects were also brought about by ultraviolet irradiation. Irradiated methyl linolenate also reduced the size of the fully formed spindle of the eggs of *Lytechinus variegatus*.

It is suggested that at least a part of the action of ultraviolet irradiation is mediated through the formation of lipid peroxides.

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THE EFFECT OF INCUBATION TEMPERATURE ON THE CELL SIZE OF *TETRAHYMENA PYRIFORMIS*

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The adaptation of higher organisms to environmental factors such as pressure, temperature, salinity, light and other ecological parameters is well known. The roots of such behavior often rest in the organization of the whole organism, an organ system or an individual organ, and may be reflected at the cellular level. The present report is concerned with the response of a unicellular system, the ciliate *Tetrahymena pyriformis*, to various incubation temperatures. Although many different micro-organisms have been available in sterile and defined media there has been little work to establish the effect of incubation temperature on the general physiological behavior of the organism. *A priori*, it is reasonable to believe that various subcellular systems will respond differently when cells are grown at different temperatures. Since cell growth and division are made up of sequentially ordered processes which most likely have different temperature coefficients, some response to temperature would be expected. Cellular adaptation to environmental factors can have broad implications. The differential control of cellular processes is a highly desirable achievement. That incubation temperatures offer an excellent area for exploration will become obvious from the experiments which are reported here.

EXPERIMENTAL

Tetrahymena pyriformis (HS) were grown in one per cent proteose peptone broth (Difco) in 500 cc Erlenmeyer flasks provided with cuvette sidearms. The sidearms permitted one to monitor the growth without the danger of contamination which normally accompanies other sampling procedures. Growth was followed with a Klett colorimeter and all experiments were carried out on cells that were taken from the early portions of the logarithmic phase of growth at a specific optical density. Not more than 150 cc of medium were

[1] Supported by National Science Foundation Grant No. 2490.

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used in the 500 cc flask so that the unfavorable surface to volume ratios normally encountered in test tubes were avoided and O_2 diffusion was not a limiting factor.

The cultures were allowed to adapt to three temperatures, 10°, 20° and 30°C for a period of one month by making serial transfers at the time the cultures reached the middle of the logarithmic phase of growth. Shorter adaptation periods may be equally effective but were not tried. Adapted cultures were sampled for two measurements: (i) the respiratory rate per one million cells, Q_{O_2} (10^6 cells), and (ii) the cell volume distribution. The respiration rates were determined in a Warburg respirometer on cells washed in $M/15$ sodium phosphate buffer and provided with an exogenous source of glucose. The concentration was less than one half million cells per cubic centimeter of fluid in the Warburg vessels. The glucose was added from the sidearm of the vessel after equilibration. All respiration measurements were made at 20°C immediately after the cells were removed from their respective incubation temperatures.

Cell volumes were determined in the following way. The *Tetrahymena* were centrifuged gently and a drop of the concentrated cells was placed on a scrupulously cleaned microscope slide. The slide had previously been equipped with a set of parallel glass fibers taken from a bundle of glass wool. A small drop of cells was placed over the fibers and a small chip of a glass coverslip was lowered into position. This operation was carried out under a binocular dissecting microscope. Excess fluid was removed from under the coverslip until the coverslip was resting firmly on the glass fibers. This was established in two ways. (i) Slight lateral movements of the coverslip caused the fiber to rotate, and (ii) the movement of cells beneath the fiber was prohibited if the coverslip and slide were in firm contact with the fibers. A bundle of fine glass wool was found having uniform fibers with diameters of 8 microns. The fiber diameter was such that all the cells were compressed and fairly motionless. It was not necessary for the fibers to be uniform since they were cylindrical and could readily be measured microscopically. Living cells mounted in this way were photographed using a 8 mm microscope objective and a 35 mm camera attachment. The pictures were taken with a large number of cells per field and included a section of the glass fiber. These photographs made it possible to determine the area and depth of the cells in the mount and from these the individual cell volume and surface area could be calculated. A series of pictures were taken of cells grown at each of the three temperatures, 10°, 20° and 30°C. The cell volumes and surface areas were determined from projections of these pictures by the use of a planimeter.

RESULTS

Fig. 1 shows the cell volume distributions of cells grown at three different temperatures, namely, 10°, 20° and 30°C. First it should be noted that the cell volume increases as the incubation temperature decreases. The average

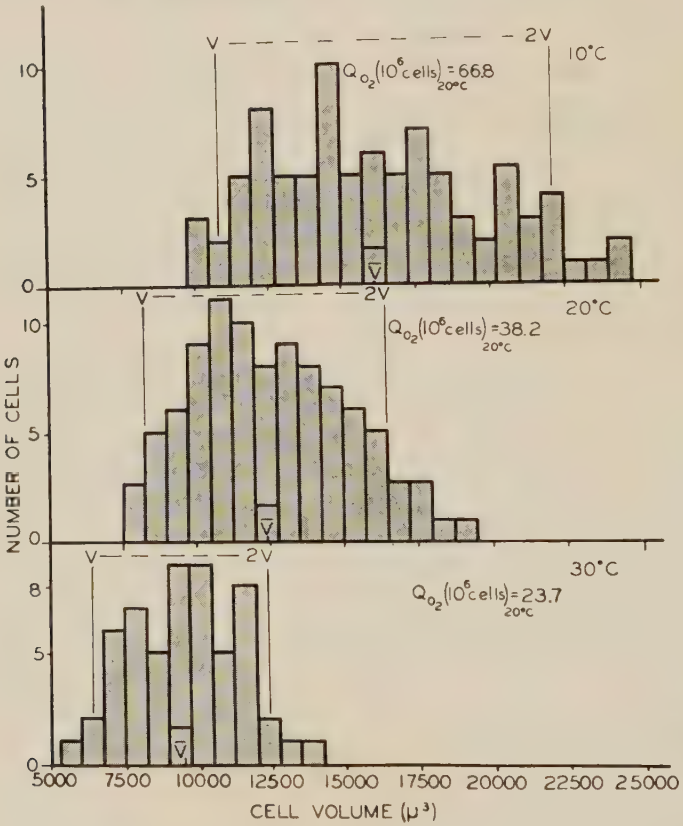

Fig. 1.—Frequency distributions of cell volumes for cells grown at three different temperatures.

volume is indicated by $\overline{V}$ and is 9375 μ^3 for the cells grown at 30°C, 12,350 μ^3 for the 20° cells, and 16,250 μ^3 for the cells grown at 10°C. The Q_{O_2} (10^6 cells) for each cell range is also listed and shows that the large cells have an average value of 66.8, while the intermediate and small cells have values of 38.2 and 23.7 respectively. The line drawn between V and $2V$ on each of the histograms indicates the range of the doubling volume of the cell. As would be expected the range of sizes falls very nearly between these two lines on each of the histograms. At both extremes of the histograms there are a few

cells that are extra large or extra small. This may have both an instrumental and real basis.

Population growth curves done on these cultures by the use of a Klett colorimeter were consistent but can have only a relative meaning in that a colored medium (proteose peptone Difco) loses color with growth of the organism. The growth curves are thus the sum of optical density (O.D.) increase due to cell number increase and O.D. decrease due to the loss of color with cell growth. The relative instantaneous growth constants (k) have values whose ratio to each other will be 4:2:1 for the cells grown at 30°, 20° and 10°C respectively. The cells at 30°C have a generation time of about three hours.

DISCUSSION

A few extensive studies on the effect of incubation temperature on such parameters as cell size and metabolism are known to the authors. Two papers by Popoff [7, 8] showed that cell size in *Paramecium* and *Frontonia leucas* was greater for the organisms grown at 17° than those at 26°C. Margalef [6] has performed a series of experiments on *Scenedesmus obliquis* and *Chlorococcum infusionum* in which the effect of temperature was found to be the primary cause influencing the cell size. His research was directed toward understanding the role of cell size, shape, and metabolism in nature. Although Margalef's work was done on photosynthetic organisms there is little reason to believe that the phenomena he observed are different from those which are reported here. The increase in cell size with a decrease in incubation temperature seems to be common to the cases mentioned above. The work of Christophersen and Precht [2] on yeast at first appears to contradict our findings. These workers found that cell volumes and dry weights were greater in cells grown at 41°C than those grown at 20°C. Temperatures lower than 20°C were not used and it could be argued that volume would again increase at low temperatures. It would be interesting to know if the cultures at 41°C were above the "optimum growth temperatures". To answer this would require data at a whole series of incubation temperatures below 20°C and a clear definition of what is meant by "optimum growth temperature". Tentatively one could consider it to be the temperature at which the growth rate in numbers was maximal or at which the generation time was shortest. However, since cell volume may vary also with temperature, the "optimum growth temperature" may not correspond when different criteria of growth are used to measure it, namely, numbers, minimum genera-

TABLE I. *Effect of incubation temperatures on cellular properties.*

$\bar{V}$ average volume, $\bar{S}$ average cell surface area, G.T. generation time, S/V surface to volume ratio, S/V sphere is the surface to volume ratio for a sphere of equal volume.

Incubation temperature (°C)	$\bar{V}$ μ^3	$\bar{S}$ μ^2	Q_{O_2} (10^6 cells)	Q_{O_2} (10^9 μ^3 cell V)	G.T. hr.	S/V	S/V sphere
10°	16,250	5,460	66	4.1	12	0.336	0.190
20°	12,350	4,320	38	3.2	6	0.350	0.208
30°	9,375	3,440	23	2.5	3	0.367	0.230

tion time, or total cell mass. The authors believe that further experimental work will be required to find just how meaningful this term can be.

The time and rate of a given adaptation are all important. Moving cells from one temperature to another may have an acute effect on their morphological and physiological characteristics, or a number of generations may be required before any effect can be ascertained. Finally, it is not unlikely that if some adaptation does occur it may include a certain amount of overshoot such that the cell will tend to readapt and end up reducing the initial response. Genetic factors dictate that the optimum growth temperature and cell volumes fall within a certain range [3, 5] and other factors, such as mineral salts and nutrients, incubation history and pH, may affect the range of its variability. The fact that cell volume increases on both sides of the pH optimum [4] in some cell types suggests that other factors may also alter the optimum growth conditions. Margalef explains his findings on the basis of the surface-dependent metabolic type defined by Bertalanffy [1]. Our findings also appear, in part, to agree with this concept. The surface to volume ratios (S/V) calculated for the cells grown at the three different temperatures in the experiment should be constant if a definite surface area is required per unit volume. As shown in Table I the ratios are 0.367, 0.350, and 0.336 for the cells grown at 30°, 20° and 10°C respectively. If spheres of the same volumes are considered, the calculated S/V ratios would be 0.230, 0.208 and 0.190 respectively as shown in Table I. The only possible way to maintain a constant S/V ratio with an increasing volume is by a distortion in the cell shape. There appears to be an adaptation of this type since the value of the ratios tend toward constancy. The Q_{O_2} (10^6 cells), as would be expected, increases with cell volume. The Q_{O_2} on a volume basis also appears to be higher for cells grown at a low temperature. This may be a reflection of a temperature-induced concentration change or the density of enzymatic systems.

Other possibilities might be a change in the properties of the enzyme as such or a change in the pathway of major reactions. Considerably more data will be required before any one of these possibilities can be ruled out.

A restatement of obvious factors which control the steady state range of cell volume and how they interact will illustrate how the S/V ratios could, in part, be the basis of these phenomena. One assumption is necessary, namely, that the cell volume will increase until the rate of oxygen diffusion across the available surface becomes limiting. Two other conditions which are derived from what is known are as follows: first, at constant temperature the rate of respiration per cell tends to remain constant, and second, a change in the incubation temperature will change the rate of respiration per unit volume. It follows that if the temperature is raised an increase in respiration rate per unit volume will occur. A decrease in volume must occur if all of the respiratory machinery is to be used since the diffusion rate per unit of surface will not be sufficient to supply the total system. On the other hand, if the temperature is lowered the rate of utilization per unit volume will decrease, and by the first assumption the volume will increase up to the point where the diffusion rates per unit area are again limiting. The magnitudes of the Q_{O_2} do not bear out these contentions, however, since if diffusion were limiting and a linear function of the amount of surface available, one would expect that the Q_{O_2} of cells twice the volume of the small cells would be less than twice the value found for the small cells. The values shown for the large cells with twice the surface area of the small cells are nearly three times that of the small cells. The distortion of cell shape and the concentration phenomenon mentioned earlier might be the explanation for this, and one must conclude that, although surface-dependent metabolism may be a large factor in the behavior of cells to incubation temperatures, at our present state of knowledge it cannot explain the findings completely.

Fig. 1 contains information of another sort. The shape of the histograms has a specific relationship to the growth curve of the individual cell. If one grants that the generation time of the organisms is constant during the logarithmic phase of growth for any given set of conditions and that the cell divisions within a rapidly growing culture are distributed randomly with respect to time, the number of cells at a particular size will be a function of the length of time that an individual cell spends at that size. A similar approach has been used by Walker [9] to determine the rate of synthesis of nucleic acids as a function of the cellular generation time. The histograms presented here do not contain a large enough sample to carry out reliable estimates of the growth curve of an average individual *Tetrahymena* at

each of the temperatures measured. The fact that the doubling volume has the proper relation to the histograms is highly suggestive that an approach of this kind would be profitable. Furthermore, if a larger sample size would not appreciably change the shape of the histograms, one could conclude, by applying Walker's method, that the growth in volume of the individual *Tetrahymena* is essentially linear. This approach is being extended to include the growth of the individual nucleus, as well as the individual cell volume, as a function of temperature.

SUMMARY

The growth of *Tetrahymena pyriformis* at three different incubation temperatures, 10°, 20° and 30°C, results in three classes of cell size having average volumes of 16,250 μ^3, 12,350 μ^3, and 9375 μ^3 respectively. The frequency distributions of each class were determined, and they indicate that the differences in the averages are not merely the result of weighting the distribution with any one cell volume since there is a shift in the entire range of the volume distributions. The Q_{O_2} (10^6 cells) were also measured. The larger cells have the higher rate of oxygen consumption when measured at 20°C, having Q_{O_2} (10^6 cells) of 66, 38 and 23 respectively. The surface to volume ratios were determined as this ratio was considered to be one factor which may govern the change in cell size with temperature. The ratios tend to remain constant. The size change cannot be entirely attributed to surface-dependent metabolism.

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ELECTRON MICROSCOPE OBSERVATIONS ON THE STRUCTURE OF SEBACEOUS GLANDS

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In the upper regions of the outer root sheaths of developing hair follicles, cell division and differentiation give rise to buds of sebaceous cells. The present report is concerned with some incipient morphological changes in these cells which were observed under the electron microscope.

METHODS

Skin from mice 1–3 days old was fixed in 1 per cent osmium tetroxide at pH 7.4 [3] for 2 hours, and dehydrated and embedded in *n*-butyl methacrylate. Ultra-thin sections were supported on carbon films and examined in a Siemens Elmiskop I electron microscope at 80 kv.

RESULTS AND DISCUSSION

Fig. 1 shows the appearance of several cells in a sebaceous bud. Their cytoplasms contain numerous almost circular profiles (*L*) about 0.5 μ in diameter which appear to be "empty" except for a small amount of debris. Usually these profiles are enveloped by one or more smooth membranes, examples of which can be seen in Figs. 2 and 3. At late stages of differentiation the cytoplasm becomes packed with these profiles.

In addition to the profiles, small dense mitochondria (*Mi*) are also to be seen in each cell (Fig. 1). These mitochondria usually contain one or more opaque granules which vary between 400 and 800 Å in diameter (Figs. 1, 2). Up to five such granules have been observed in a sectional profile of one mitochondrion. The non-sebaceous cells of the outer root sheath do not possess the "empty" circular profiles. Moreover, their mitochondria do not contain any opaque granules (Fig. 2).

Membranes of endoplasmic reticulum studded with granules [4], were rarely seen in either the sebaceous or the non-sebaceous cells of the outer root sheath. Indeed, their rarity is a consistent feature of the holocrine cells of all the differentiated layers of the hair follicle.

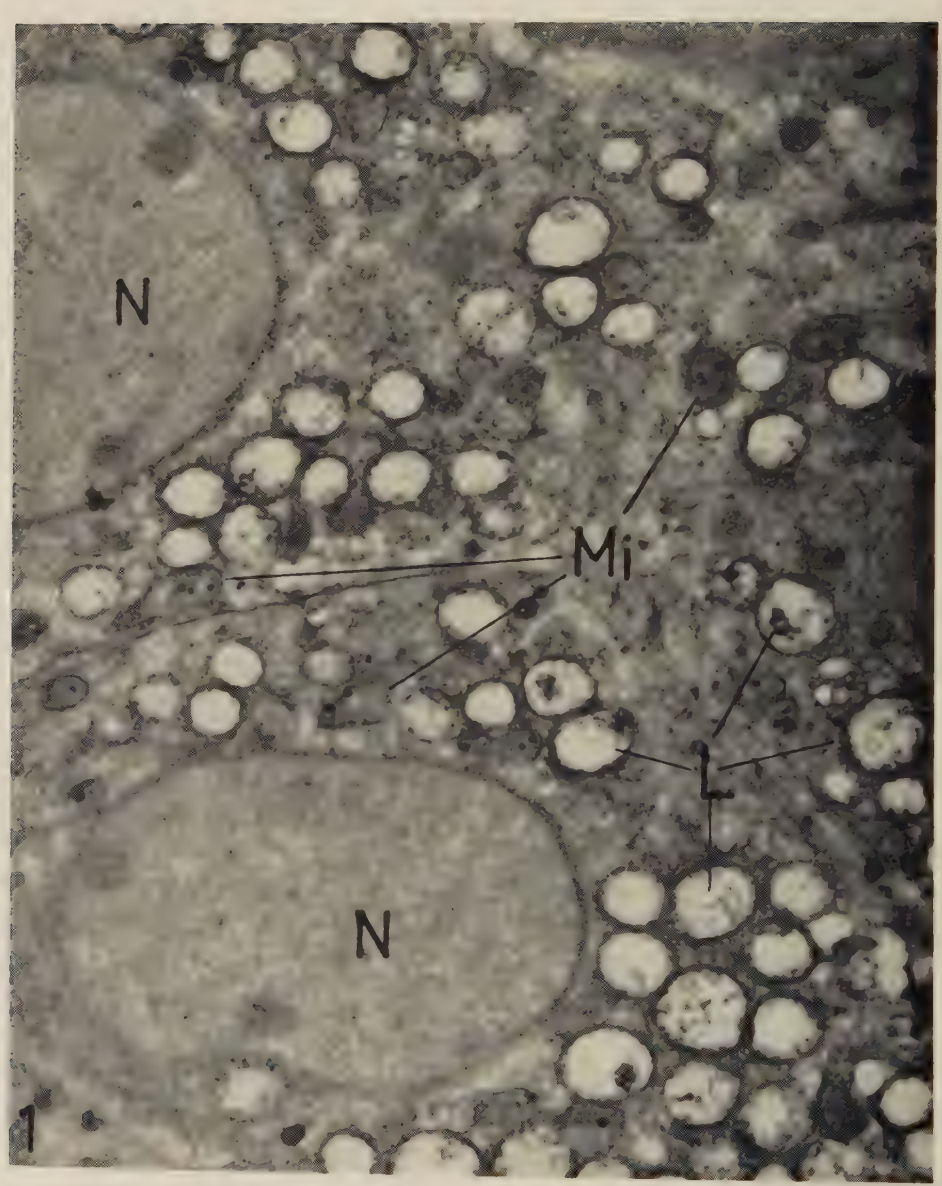
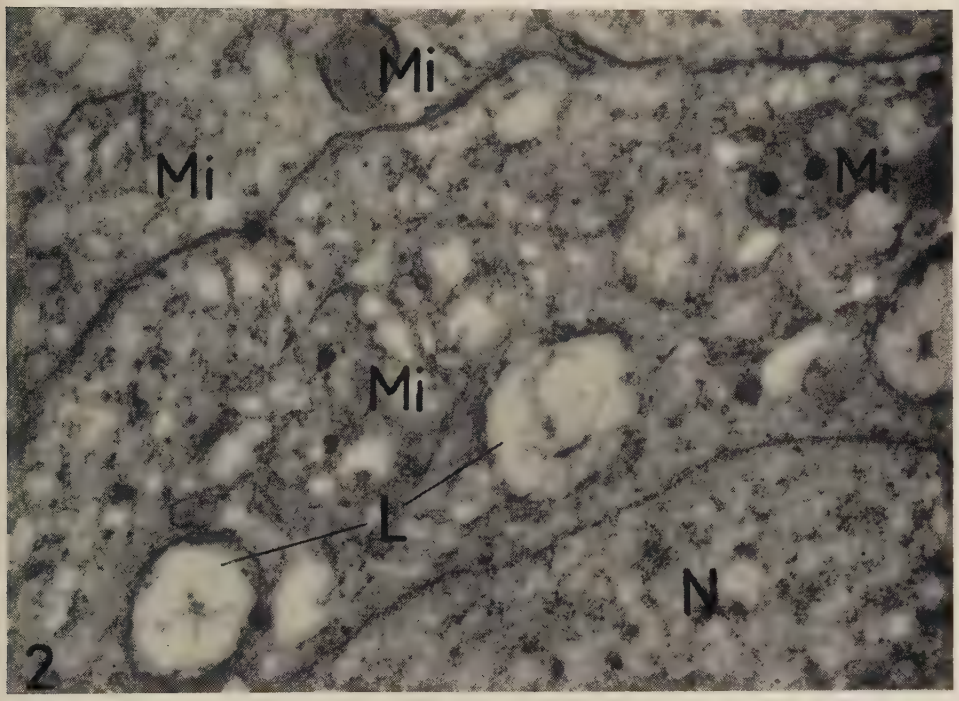


Fig. 1. A group of three cells of a sebaceous bud is shown in which circular profiles (lipid droplets) and mitochondria containing dense granules are present. *N*, nucleus; *Mi*, mitochondria; *L*, lipid droplets. Magnification ×15,000.



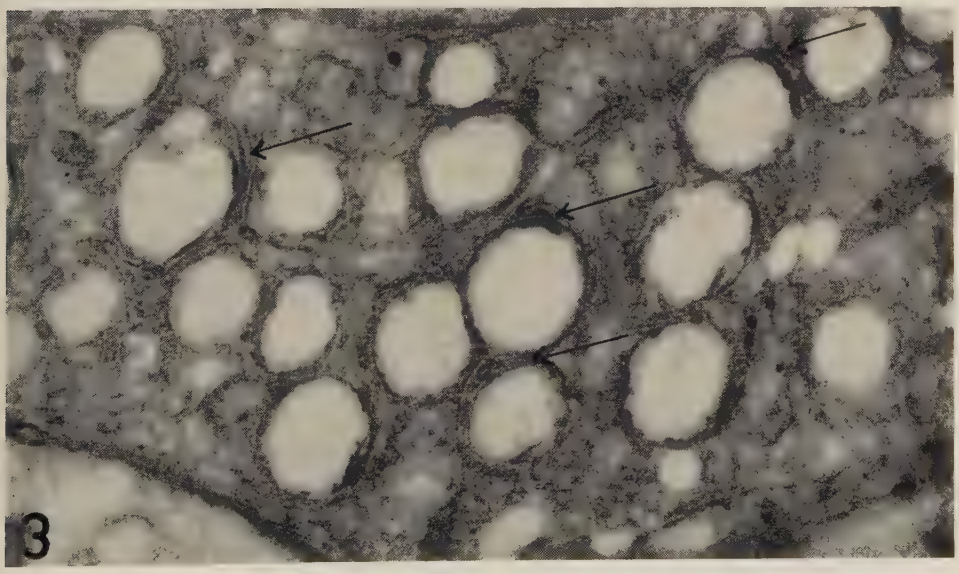


Fig. 2.—Part of a sebaceous cell which contains both the circular profiles and mitochondria. Adjacent to it (top left-hand of micrograph) is a non-sebaceous cell with two mitochondria which do not contain any dense granules. Magnification $\times 30{,}000$.
Fig. 3.—A region of the cytoplasm in a sebaceous cell showing many circular profiles (lipid droplets) separated from one another by smooth membranes (arrows). Magnification $\times 25{,}000$.

The morphological features described here enable the very earliest stages of the sebaceous transformation to be recognized in the cells of the outer root sheath. The "empty" circular profiles seen in cells which are differentiating to the sebaceous type must correspond with the perinuclear sudanophil and osmiophobic lipid globules which were observed in young sebaceous acini and also in sebaceous cells which regenerated from the outer root sheath after the treatment of mouse skin with methylcholanthrene [1, 2]. Their "empty" appearance in electron micrographs must have resulted from the removal of most of the lipid during the fixation and embedding procedure.

The presence of opaque granules in the mitochondria of these cells is of interest. Their different appearance in this respect, as compared with the mitochondria in other differentiating cells of the hair follicle, suggests a close relationship with the process of the formation of lipid in the sebaceous cells. Further investigation is necessary in order to establish whether or not these mitochondria are undergoing a transformation into lipid inclusions.

Opaque granules have been observed previously in the mitochondria of many types of cells [6, 7, 8], and in addition Sheldon and Zetterqvist [5] have put forward evidence to suggest that keratohyalin granules develop within mitochondria.

SUMMARY

The cells of the outer root sheath of hair follicles of young mice undergoing a sebaceous transformation were observed under the electron microscope. "Empty" circular profiles, presumably derived from lipid inclusions, and mitochondria containing dense granules 400–800 Å in diameter, were seen in the cytoplasm of these cells. These features distinguished the sebaceous cells in the outer root sheath.

This study was carried out at the Anatomy School, Cambridge. The author is indebted to Professor J. D. Boyd, Anatomy School, Cambridge, and Dr. V. E. Cosslett, Cavendish Laboratory, for research facilities.

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ELECTRON MICROSCOPE OBSERVATIONS ON THE GLASSY LAYER OF THE HAIR FOLLICLE

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The glassy layer (glassy membrane, vitreous membrane) is situated around hair follicles between the outer root sheath and the connective tissue of the surrounding dermis. Under the light microscope it appears as a thin hyalin layer. The staining reactions of the glassy layer and its relationship to the follicle have been described [5, 6, 15]. The present paper is concerned with the submicroscopic structure of this layer as revealed by the electron microscopy of ultrathin sections of osmium-fixed material.

METHODS

Pieces of dorsal skin (2 mm × 2 mm) from decapitated mice 1–3 days old, and from adult guinea pigs, were fixed in 1 per cent osmium tetroxide at pH 7.4 [7] for 4 hrs. The specimens were washed in Ringer, dehydrated and embedded in *n*-butyl methacrylate in the customary manner.

Ultra-thin sections were cut on a microtome [4] and mounted on carbon films on copper grids. They were examined in a Siemen's Elmiskop I fitted with a 25 μ objective aperture at an accelerating voltage of 80 kV.

RESULTS

The glassy layer during follicle development.—In sections of skin from 3-day-old mice examined under the electron microscope, young hair follicles were seen which were completely encased in a network of closely adjoining fibroblasts (Figs. 1, 2, 3). The cytoplasm of many of these fibroblasts was filled with mitochondria and elements of the endoplasmic reticulum. Beneath this network of fibroblasts the basal borders of the outer root sheath cells were overlaid by an electron-optically homogeneous submicroscopic layer about 200 Å thick (Fig. 2). As follicles continue to grow and elongate, their glassy layers also develop. Young fibrils with a uniform diameter of about 200 Å appeared in these regions in close association with the homogeneous layer (Fig. 3).

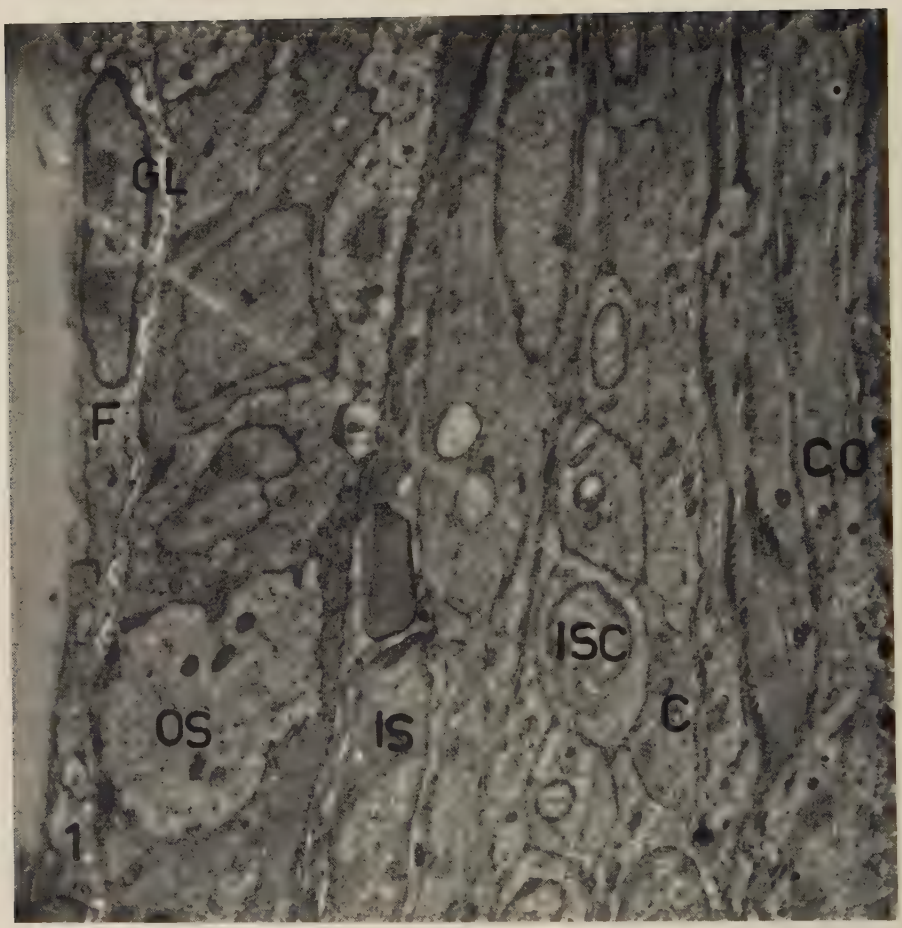
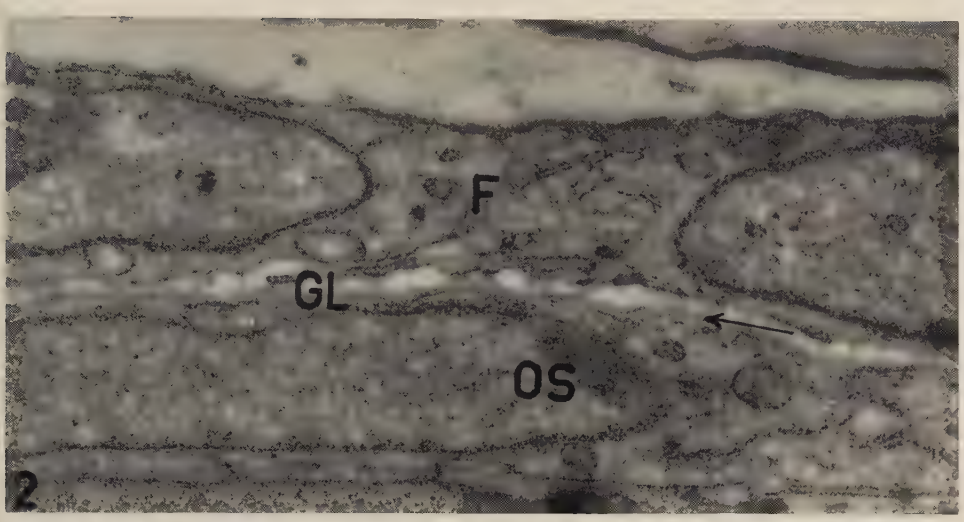


Fig. 1.—Survey picture of main layers of the upper bulb region of a hair follicle from a 3-day-old mouse, after keratinization has begun. Note the outermost layer of fibroblasts (*F*) separated from the outer root sheath (*OS*) by a space in which the glassy layer (*GL*) develops. Further into the follicle from the outer root sheath, the differentiated layers of the inner root sheath (*IS*), and its cuticle (*ISC*), hair cuticle (*C*) and cortex (*CO*) can be distinguished. Keratin fibrils are present in the cells of the cortex. Magnification ×6000.

The glassy layer of fully-formed follicles.—The hair follicles of adult guinea pigs were studied. From their general appearance under the electron microscope it was apparent that the follicles were in the late anagen phase. Because of the dense collagenous dermis in adult skin some difficulty was experienced in obtaining sections of areas of follicles which were optimally preserved. In favorable sections which were orientated along the longitudinal axis of a



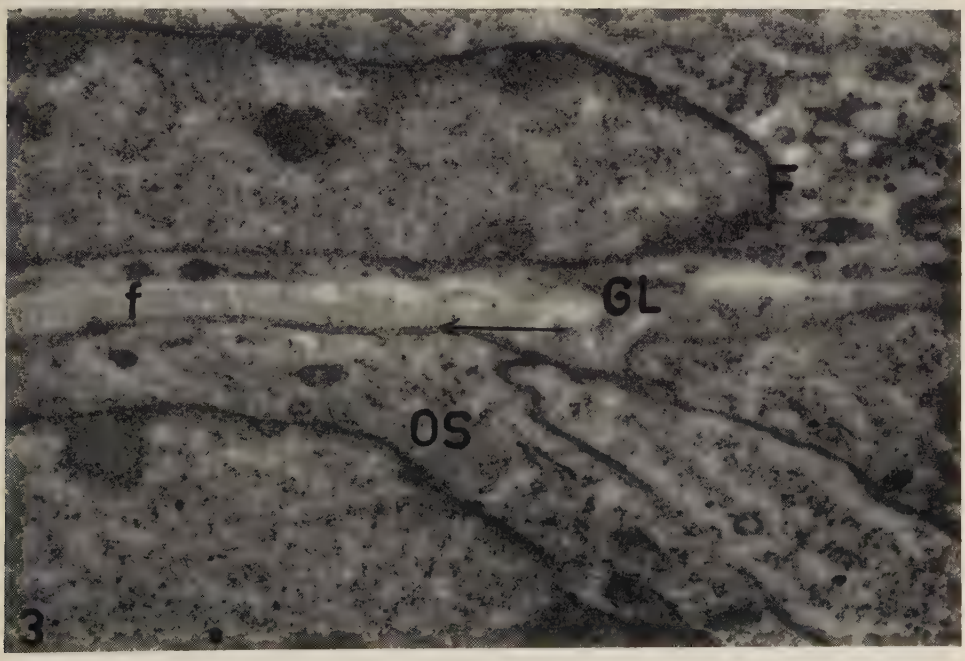
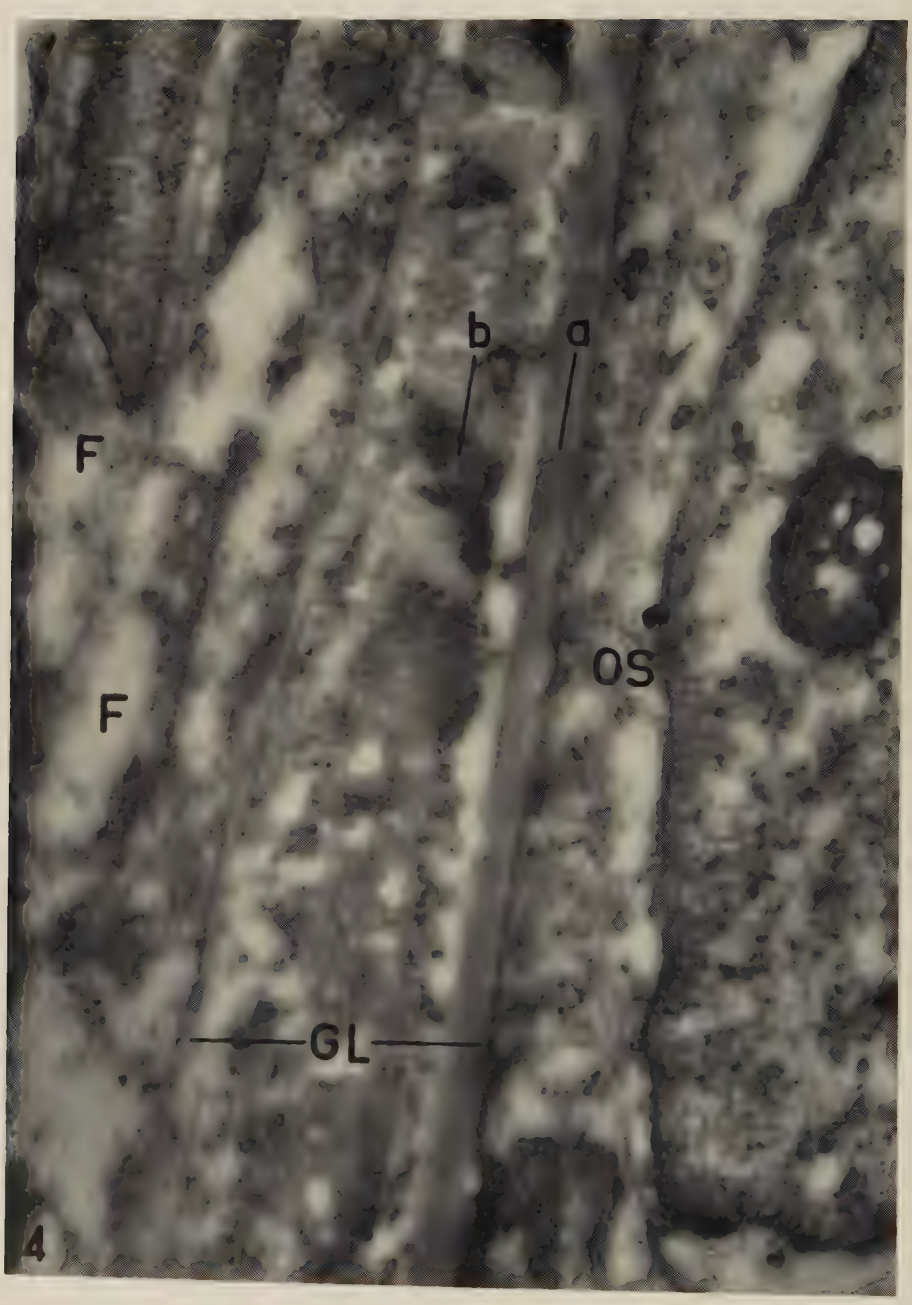


Fig. 2.—Cross-section of a hair follicle from a 3-day-old mouse before keratinization has begun. Note two interlocking fibroblasts (*F*) which are part of a complete layer which surrounds the outer root sheath (*OS*). The glassy layer (*GL*) develops in the space between these cells which is partly filled with electron-optically homogeneous material (arrow). Magnification × 22,000.
Fig. 3.—As in Fig. 2 but a later stage with fibrils (*f*) appearing in the glassy layer. Note the homogeneous layer (arrow) which covers the basal borders of the cells of the outer root sheath (*OS*). Magnification × 22,000.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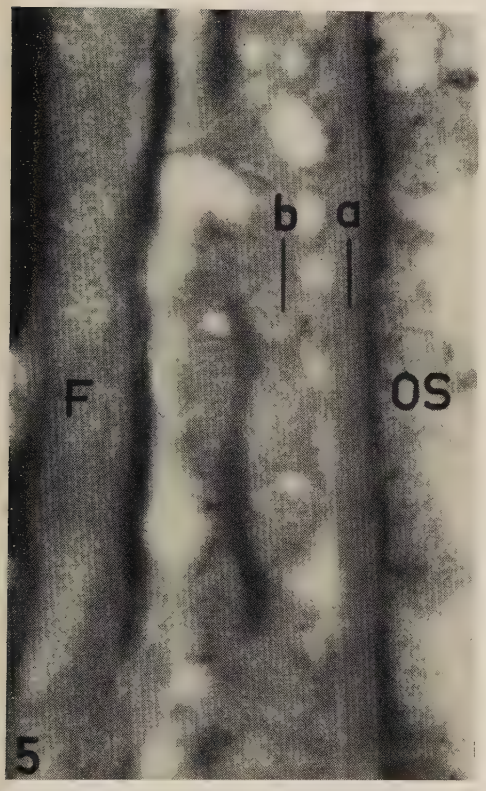

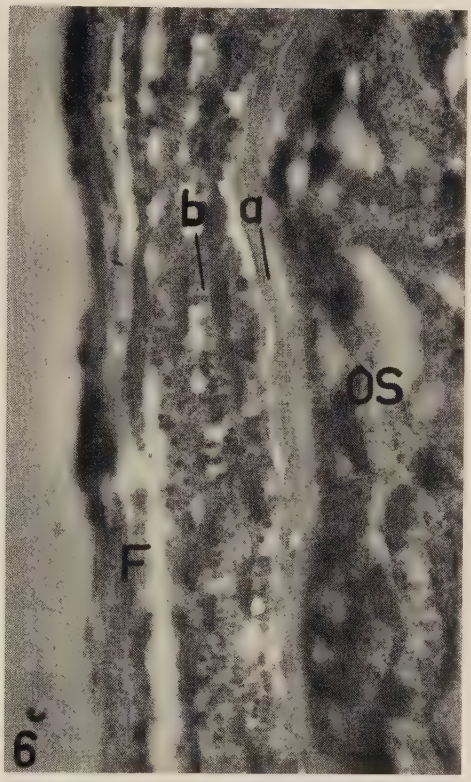

Fig. 4.—Longitudinal section of a follicle of a guinea pig, showing the structure of the glassy layer (*GL*). Fibrils are in longitudinal array (*a*) next to a cell of the outer root sheath (*OS*), whereas in (*b*) the fibrils are orientated at right angles to those in (*a*). Two fibroblasts (*F*) are situated at the limit of the glassy layer. Magnification ×25,000.

Fig. 5.—A longitudinal section of a follicle of a guinea pig showing the 500 Å periodicity of the fibrils in the inner array (*a*) of the glassy layer. This feature is not prominent in Fig. 4. Magnification ×30,000.

Fig. 6.—As in Figs. 4 and 5, showing the structure of the glassy layer of a guinea pig follicle, including the 500 Å periodicity in the longitudinal array of fibrils (*a*) of the glassy layer. Magnification ×20,000.

follicle, the following features were seen in the glassy layer at a level corresponding to the keratogenous zone of the cortex.

(*a*) An inner array of fibrils which lies adjacent to the basal borders of the cells of the outer root sheath; these fibrils are orientated parallel to the longitudinal axis of the follicle (Figs. 4, 5, 6). They are about 500 Å in diameter, extremely long and have an axial periodicity of the order of 500 Å.

(*b*) An outer array of fibrils on the dermal side of the first and in close

association with it. The fibrils of this layer are all orientated at right angles to those of the inner array (Figs. 4, 5, 6). Occasionally they are grouped into bundles.

The long collagen fibrils of the general dermis also showed axial cross-striations at periods of about 500 Å, but in contrast to the fibrils of the glassy layer, they were 800–1000 Å in diameter.

The fibroblasts which are so prominent around young follicles are still to be found around the follicles of adult skin (Figs. 4, 5) but they are greatly attenuated. Consequently they were seen less frequently under the electron microscope.

DISCUSSION

The glassy layer is fairly prominent in guinea pig follicles [8] whereas in mice it is thin [2], although Wolbach [15] has described marked changes in the latter during cycles of hair growth.

The glassy layer of guinea pigs was stated by Stöhr [11] to consist of two components, one proximal to the outer root sheath, which he assumed to be of epithelial origin, and an outer component from connective tissue. Montagna [6] has presented histological evidence which suggests that in the human scalp the glassy layer has a dual origin. He also found that one layer was a mixture of fibrils and of cytoplasmic processes from the cells of the outer root sheath. In the present study of guinea pig follicles both the structure of the glassy layer and also its relationship to the outer root sheath are clearly defined.

The presence of collagen in the layer has been accepted from its known affinity for collagen stains [6, 15]. It also stains with silver [5, 6, 15], a property which histologists ascribe to the presence of reticulin. The "basement membranes" of many epithelia stain in this way. The present demonstration of collagen fibrils in the glassy layer, therefore, bears out the opinion of several authors that reticulin is basically collagen [1, 3].

The submicroscopic and electron-optically homogeneous layer, which was observed next to the outer root sheath of young follicles, is similar to other membranes of this type which are present beneath most epithelia so far studied under the electron microscope [10]. Although its chemical nature is not known, it could be a mucopolysaccharide complex [14]. At least some of the fibrils of the glassy layer develops within this material, and it would seem that it may be responsible for the marked affinity for silver displayed by the fibrils and their positive reaction towards the PAS stain.

The electron microscope has not only revealed the fibrillar nature of the glassy layer but also cross-banding in the fibrils which is characteristic of mature collagen. However, it should be noted that the period observed (500 Å) is considerably less than the accepted value (640 Å). This discrepancy has been observed in human skin in thin sections [9] and remains unexplained.

The diameter of the fibrils of the glassy layer is only about 500 Å whereas the fibrils which comprise the fibres of the general connective tissue of the dermis are 800–1000 Å in diameter. Presumably the fibrils of the glassy layer are produced by the fibroblasts closely associated with the follicle. Many of the fibroblasts in young follicles are densely filled with mitochondria and endoplasmic reticulum, an appearance which is in accord with high activity. In view of the smaller diameter of the fibrils in the glassy layer, their growth must terminate at an earlier stage than in the general dermis.

It is of further interest to note how the structure of the glassy layer closely resembles that of the basement lamellae of larval amphibian skin investigated by Weiss and Ferris [12, 13, 14]. The glassy layer however, consists only of two sheets of collagen fibrils orientated at right angles to one another, whereas the fabric of collagen fibrils investigated by these authors was multilayered.

SUMMARY

The structure of the glassy layer of hair follicles of young mice and adult guinea pigs was studied in the electron microscope.

The glassy layer of a fully-formed follicle of the guinea pig consists of long fibrils of collagen-type which are about 500 Å in diameter. They are organized into two arrays. An inner array adjoining the basal borders of the cells of the outer root sheath, and parallel to the follicle length, and an outer array orientated at right angles to the first.

In the developing follicles of young mice the fibrils of the glassy layer are about 200 Å in diameter. They develop in the space between the outer root sheath and a layer of fibroblasts which surrounds each follicle.

This study was carried out at the Anatomy School and Cavendish Laboratory, Cambridge. The author wishes to thank Professor J. D. Boyd and Dr. A. Hughes of the Anatomy School for their interest, and Dr. V. E. Cosslett and Mr. R. W. Horne of the Cavendish Laboratory for electron microscope facilities.

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QUELQUES ASPECTS HISTOLOGIQUES DE *LIMNOCNIDA TANGANYICAE*

OBSERVATIONS DE COUPES ULTRAFINES AU MICROSCOPE ÉLECTRONIQUE

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Si l'examen de coupes ultrafines au microscope électronique a fait faire des progrès considérables à nos connaissances sur la structure intracellulaire, ces méthodes ont, par contre, été peu employées pour résoudre des problèmes histologiques.

Par la simplicité de leur structure didermique et la présence d'organites aisément identifiables tels que les nématocystes et les vacuoles de réserve, les Coelentérés offrent un matériel de choix pour l'étude histologique au microscope électronique.

Nous nous sommes proposés d'étudier l'histologie de l'anneau urticant, du canal circulaire, du velum et des tentacules de *Limnocnida tanganyicae*, Limnoméduses, dont l'un de nous vient de terminer l'étude histologique par les méthodes classiques [7].

Les animaux récoltés dans les eaux du lac Tanganyika ont été fixés dans le fixateur d'Altmann (acide osmique 2%, bichromate de potassium 5 %). Les fragments à étudier ont été enrobés dans du méthacrylate de butyle selon la méthode courante et coupés à l'aide d'un microtome Porter Sorvall. Les coupes obtenues avaient une épaisseur variant entre 150 et 250 Å. Les préparations ont été examinées au microscope électronique Philips E.M. 100B du Centre de Microscopie Electronique « CEMEMIA », Uccle.

Endoderme du canal circulaire

On sait que les Coelentérés présentent deux phases de digestion successives : une phase de digestion extracellulaire et une phase de digestion intracellulaire. Chez *Limnocnida* le terme de ces processus digestifs consiste dans la formation de substances assimilables, de vacuoles protéiniques de réserve et de vacuoles excrétrices [7].

La Fig. 1 nous montre l'aspect au microscope optique d'une portion du canal circulaire et de l'anneau urticant de *Limnocnida*; on distingue vacuoles de réserve (*R*), les vacuoles digestives (*V*) et les vacuoles excrétrices (*E*).

Les Fig. 2, 3, 4 et 5 sont des micrographies électroniques de fragments de cellules endodermiques du canal circulaire. On note la présence de mitochondries (*M*) et de bâtonnets d'ergastoplasme (*Er*) entourant les inclusions digestives (*R*). Au sein de l'hyaloplasme on distingue également des vacuoles digestives (*V*) à parois souvent lobulées, des membranes intracytoplasmiques et de curieuses formations vésiculeuses très abondantes présentant en coupes une forme de U plus ou moins ouvert.

Les cellules des Coelentérés sont, nous le savons, caractérisées par leur aspect vacuolisé lié à la grande concentration aqueuse existant à l'intérieur de leurs tissus (plus de 99% pour l'ensemble des tissus de *Limnocnida*).

Les observations au microscope électronique confirment la faible densité des éléments intracytoplasmiques, notamment la dispersion des mitochondries et de l'ergastoplasme.

Mitochondries

Grâce aux récentes recherches faites au microscope électronique la structure des mitochondries nous est bien connue à l'heure actuelle. Chez les Vertébrés ces organites semblent bâtis selon un même plan, quelle que soit leur origine [1–6, 16, 17, 22, 23]. Les mitochondries des cellules endodermiques du canal circulaire de *Limocnida* présentent une structure fondamentalement semblable. Généralement ovalaires, elles apparaissent parfois sphériques. Ces variations dépendent en grande partie de l'incidence des coupes et de l'état physiologique de la cellule. Entouré d'une double paroi, le corps mitochondrial est cloisonné par un système rayonné de doubles membranes internes dont certaines semblent en continuité avec la membrane interne de l'enveloppe. Or, on sait que chez les Vertébrés ces lamelles sont généralement perpendiculaires au grand axe de l'organite et que ce n'est qu'exceptionnellement qu'elles sont en continuité avec la membrane interne de la double paroi limitante.

Les mitochondries observées dans les cellules de *Limnocnida* sont de faible

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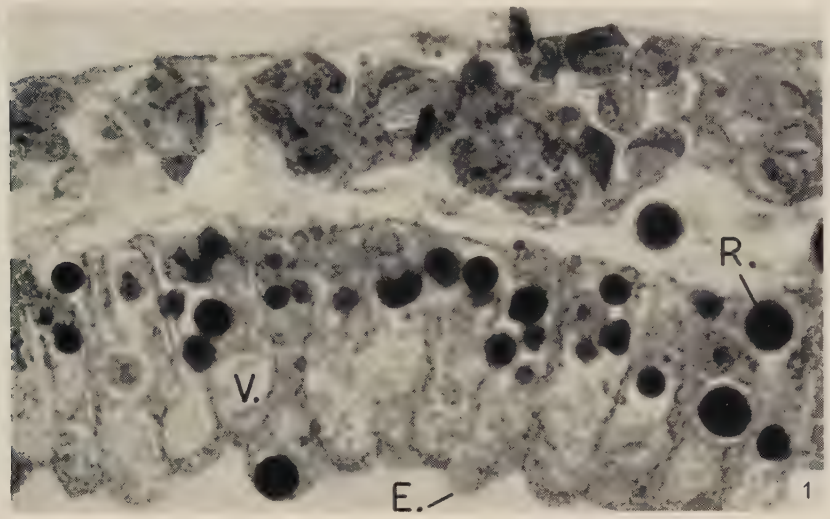
Fig. 1.—Microphotographie d'une coupe de fragment d'exombrelle de *Limnocnida* passant au niveau de l'anneau urticant et du canal circulaire. *E*, vacuoles excrétrices; *R*, sphérules digestives; *V*, vacuoles digestives. Microscope optique, objectif à immersion.

Fig. 2.—Micrographie électronique d'une vacuole digestive initiale. On remarque les fragments ingérés au cours de la digestion intracellulaire ainsi que des structures lamellaires. Grossissement 83.700 ×.


R.
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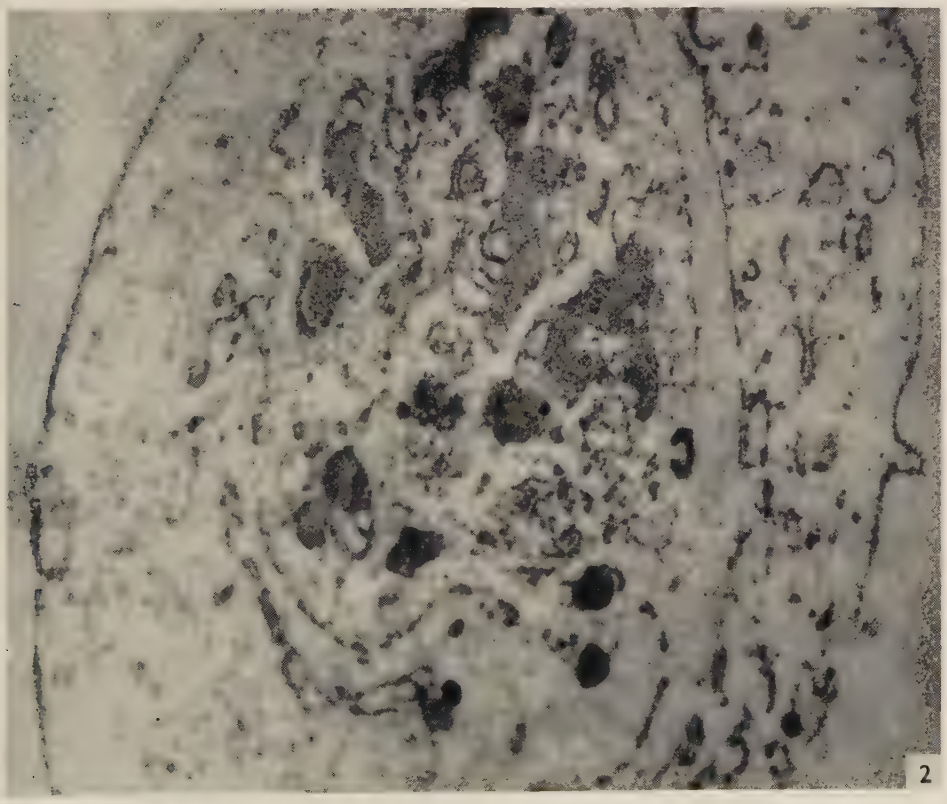
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taille (grand axe 0,10 à 0,25 μ, petit axe 0,10 à 0,14 μ) par rapport à celles des Vertébrés qui atteignent fréquemment des dimensions supérieures au micron.

Ergastoplasme

Propre à l'endoplasme des cellules sécrétrices, l'ergastoplasme est considéré actuellement comme une spécialisation locale du "reticulum endoplasmique" [16, 18].

L'ergastoplasme se présente généralement, quelle que soit l'orientation des coupes, sous la forme de structures lamellaires parallèles. Les grains de Palade répandus dans tout le "reticulum" sont particulièrement abondants au niveau des lamelles, donnant à ces structures leur aspect si caractéristique [4, 5, 6, 9, 12 et 25].

Les microsomes décrits par les biochimistes résulteraient du fractionnement de ces structures ergastoplasmiques, les grains de Palade correspondant à la portion ribonucléique des microsomes, les structures lamellaires portant vraisemblablement leur stock enzymatique [18].

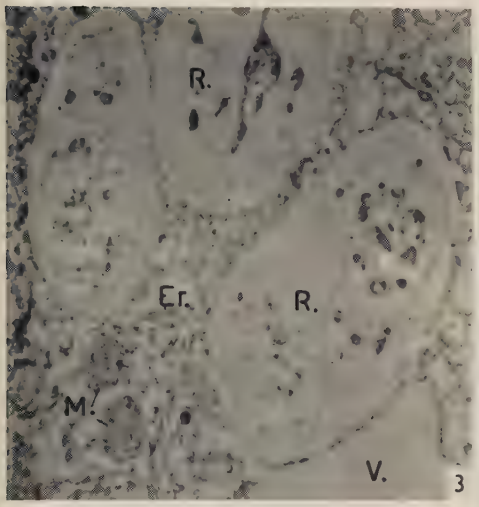
Les récentes expériences de Bernhard et Rouiller [6] semblent, de plus, démontrer l'étroite relation fonctionnelle existant entre ces formations ergastoplasmiques et le système mitochondrial.

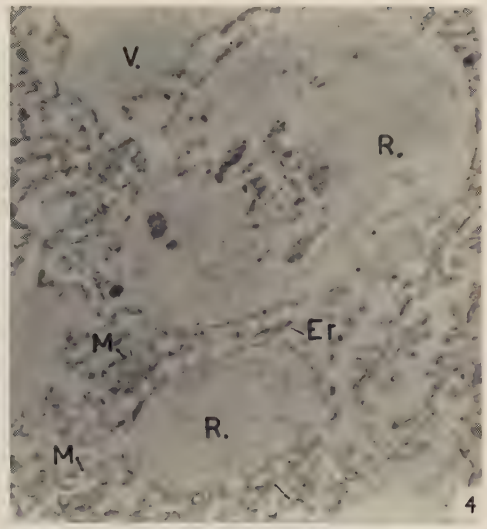
Les cellules endodermiques du canal circulaire de *Limnocnida* contiennent des formations que nous pensons être ergastoplasmiques quoique très dispersées. Ces structures sont constituées d'une série de doubles membranes assez épaisses, parsemées extérieurement de nombreux granules de Palade mesurant environ 150 Å. La matrice interne de ces éléments ergastoplasmiques apparaît plus dense que l'endoplasme environnant.

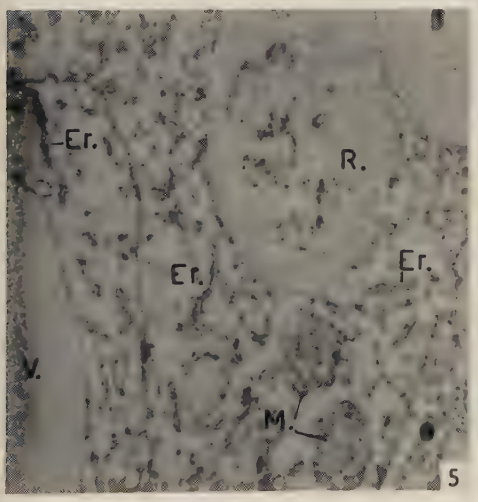
Inclusions de reserve

Ces organites protéiniques sont élaborés au sein des cellules absorbantes. Leur évolution cytologique peut se résumer comme suit : les substances provenant de la digestion extracellulaire sont absorbées par les cellules endodermiques au niveau des vacuoles digestives. Celles-ci s'agglomèrent et forment des sphères muriformes riches en acide ribonucléique qui se fragmentent ultérieurement en sphérules plus petites mais présentant les mêmes caractères. Ces sphérules vont soit être assimilées immédiatement, soit se transformer partiellement ou totalement en vacuoles de réserve [7, 21].

Les micrographies électroniques 2, 3, 4 et 5 nous montrent quelques stades de la transformation des vacuoles digestives en organites de réserve. La première correspond à une coupe passant au niveau d'une vacuole digestive







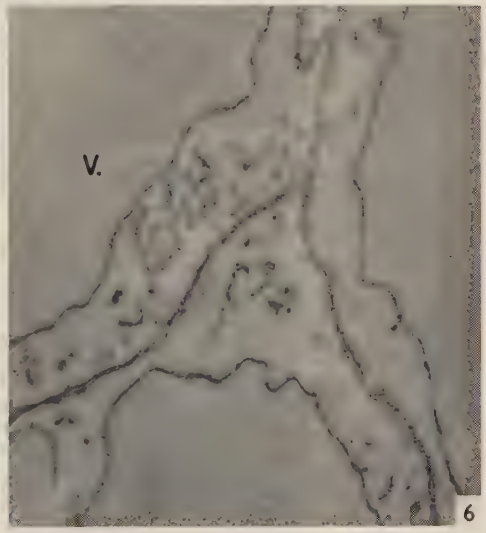


Fig. 3 et 4.—Micrographies électroniques de portions de cellules endodermiques du canal circulaire de *Limnocnida*. On distingue les mitochondries (*M*) et l'ergastoplasme (*Er*) entourant les sphérules de réserve (*R*). *V*, vacuoles digestives. Grossissements : Fig. 3, 24.700 × ; Fig. 4, 28.000 × .

Fig. 5.—Partie agrandie de la Fig. 3 montrant les détails de l'ergastoplasme (*Er*) et des mitochondries (*M*). On note la présence de formations vésiculeuses en forme de U (voir également Fig. 3 et 4). *V*, vacuoles digestives. Grossissement 34.600 ×

Fig. 6.—Limite de trois cellules endodermiques tentaculaires. Remarquer les doubles membranes cellulaires et les vacuoles (*V*). Grossissement 40.300 × .

initiale; on y distingue des fragments ingérés très denses aux électrons et de nombreuses structures lamellaires d'aspect ergastoplasmique. Les Fig. 3, 4 et 5 nous montrent par contre la structure des inclusions de réserve définitivement constituées.

A l'encontre des observations faites au microscope optique les micrographies électroniques nous montrent que ces vacuoles de réserve sont hétérogènes. En effet, elles contiennent encore des petits fragments de substances ingérées et des résidus lamellaires.

Endoderme tentaculaire

L'endoderme des tentacules de la méduse *Limnocnida* est formé de cellules épithélio-musculaires très vacuolisées. La majeure partie du volume de la cellule est occupée par une vacuole centrale, le cytoplasme étant refoulé à la périphérie, accolé en une mince pellicule tout au long de la membrane cellulaire.

La Fig. 6 nous montre les limites de trois cellules endodermiques, on distingue les doubles membranes cellulaires et les vacuoles (*V*). Le noyau de ces cellules endodermiques est ovalaire; généralement basal, il fait hernie dans la vacuole centrale et est inclus dans une mince bande cytoplasmique.

La Fig. 7 nous montre un de ces noyaux. On observe la chromatine lâchement dispersée sous forme de petits grains irréguliers, le nucléole ovalaire très dense et finement granuleux ainsi que par endroits, la double membrane nucléaire.

Examiné à des très forts grossissements (de l'ordre de 200.000 fois) le nucléole apparaît comme étant formé de granulations très serrées; plus ou moins condensées en quelques traînées, lesquelles présentent une certaine régularité et donnent à l'ensemble un aspect réticulé ou pelotonné très différent de celui décrit chez les nucléoles de Vertébrés par Bernhard et ses collaborateurs [2–6, 8].

On pourrait se demander si ce ne sont pas ces traînées apparamment réticulées ou pelotonnées qui suggèrent l'hypothèse d'un constituant filamenteux ou nucléolonema au sein des nucléoles des Vertébrés. De toute façon l'existence d'une telle structure reste à démontrer chez les Invertébrés.

Des structures fibrillaires se rencontrent toutefois dans la région périphérique du nucléole mais sont alors extrêmement ténues.

Il est généralement admis à l'heure actuelle que le nucleolonema et principalement les structures granuleuses observées à son niveau représentent la phase ribonucléique si caractéristique du nucléole, l'hétérochromatine étant analogue à la « pars amorpha » [9, 5]. Etant donné les grossissements

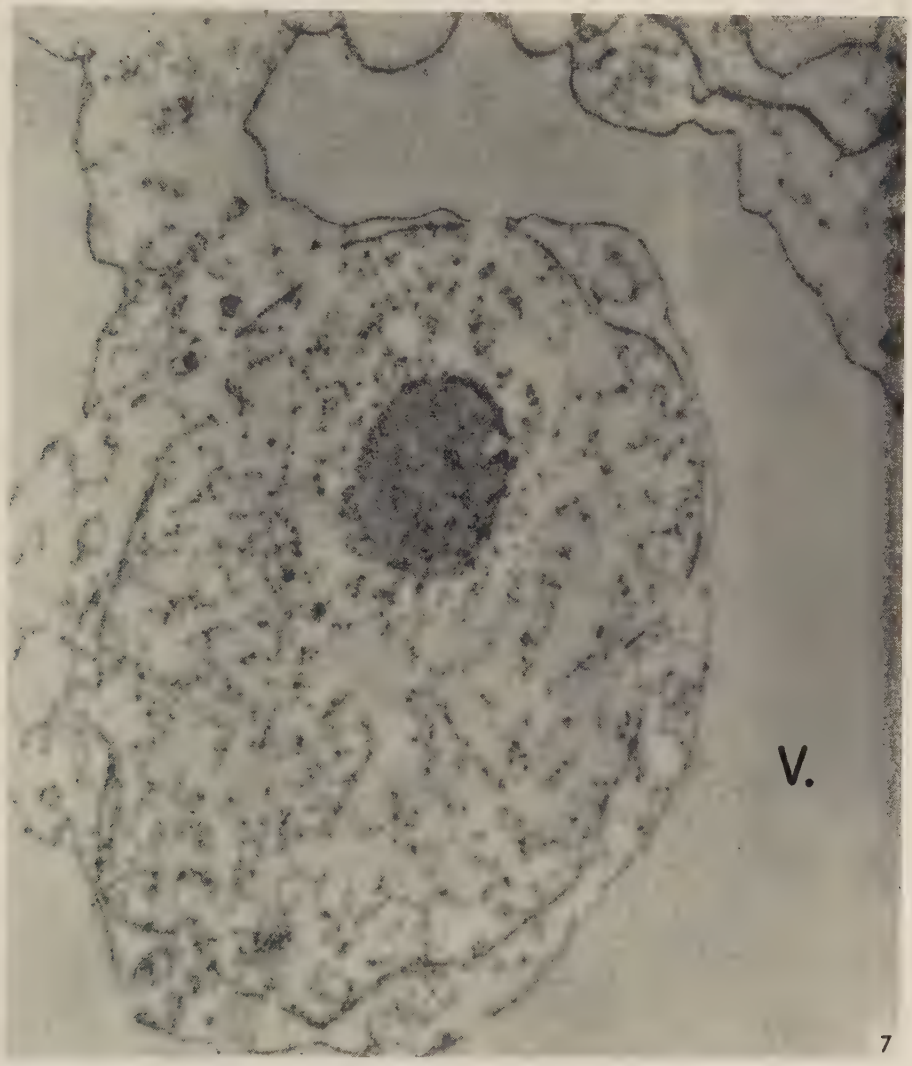


Fig. 7.—Noyau d'une cellule endodermique tentaculaire inclus dans une mince pellicule cytoplasmique. Le nucléole est finement granuleux, sa région périphérique est plus dense et peu nette. Grossissement 42.000 ×.

obtenus, les structures fibrillaires observées au niveau du nucléole pourraient correspondre à des macromolécules fibreuses d'acide ribonucléique.

La région périphérique du nucléole est plus dense et ne représente pas une limite nette. Certains éléments (granulations et fibrilles) semblent d'ailleurs se répandre dans le suc nucléaire.

Fig. 8.—Micrographie fortement agrandie du nucléole représenté dans la Fig. 7. Noter l'aspect granuleux et les structures fibrillaires périphériques. Grossissement 185.000 ×.

Musculature vélaire

Les fibres musculaires se différencient chez les Coelentérés dans le cytoplasme basal des cellules épithéliales ectodermiques et endodermiques. Chez les méduses la musculature du velum et de la sous-ombrelle est particulièrement développée et de plus, striée.

De nombreux auteurs se sont attachés à discuter la structure submicroscopique de ces muscles; pour les uns elle serait semblable à celle des muscles striés de Vertébrés, pour les autres elle serait totalement différente.

Les Fig. 9 et 12 nous montrent des muscles épithéliomusculaires du velum de *Limnocnida*. Ces fibres musculaires constituées d'un nombre relativement restreint de fibrilles (environ 20), sont caractérisées par une succession de zones alternativement sombres et claires. L'ensemble de ce système fibrillaire est homologue à un myofibrille de muscle strié de Vertébrés; sa structure semble d'ailleurs en être très voisine.

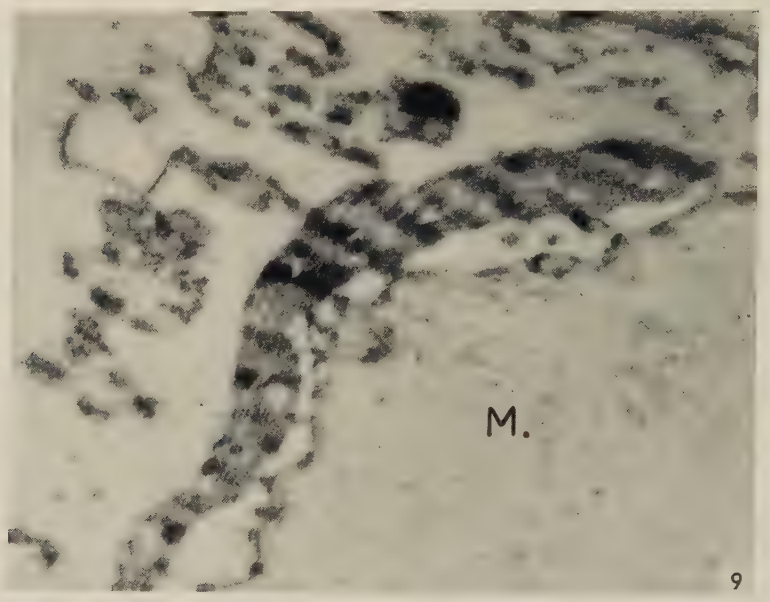
Les zones sombres pourraient en effet correspondre aux zones des muscles des Vertébrés; tout comme celles-ci elles sont partagées en deux portions par une bande moins colorée qui serait dès lors identique à la bande *H*. Les zônes claires s'apparenteraient aux zones *I*. Le disque *Z* si caractéristique du muscle de Vertébrés ne se retrouve pas sur nos micrographies, mais il est actuellement admis que c'est par cette bande *Z* que le signal de la contraction est transmis de myofibrille à myofibrille au travers d'un même muscle; on pourrait admettre que chez les Coelentérés où l'ensemble des éléments musculaires d'une cellule épithélio-musculaire correspond à une myofibrille isolée, une telle disposition n'est pas nécessaire.

L'examen au microscope électronique nous permet également de préciser la position de ces fibres. On sait en effet que si pour la majorité des auteurs les fibres musculaires des Coelentérés se trouvent au sein d'expensions basilaires à l'intérieur des cellules, d'autres tel que Holmes [13], nient une telle disposition et admettent la position extracellulaire des fibres musculaires.

Les micrographies électroniques ne laissent subsister aucun doute : la fibre musculaire se trouve à l'intérieur de la cellule, accolée à la mésoglée à laquelle elle est d'ailleurs attachée en plusieurs endroits (Fig. 9 et 11). On pourrait imaginer que ces points d'attache permettent la transmission de contraction, d'une cellule à l'autre et au travers du velum par l'intermédiaire de la mésoglée fibrillaire.

Nématocystes

L'examen de coupes ultrafines des tentacules et de l'anneau urticant de *Limnocnida* nous permet d'apporter quelques précisions sur la structure de



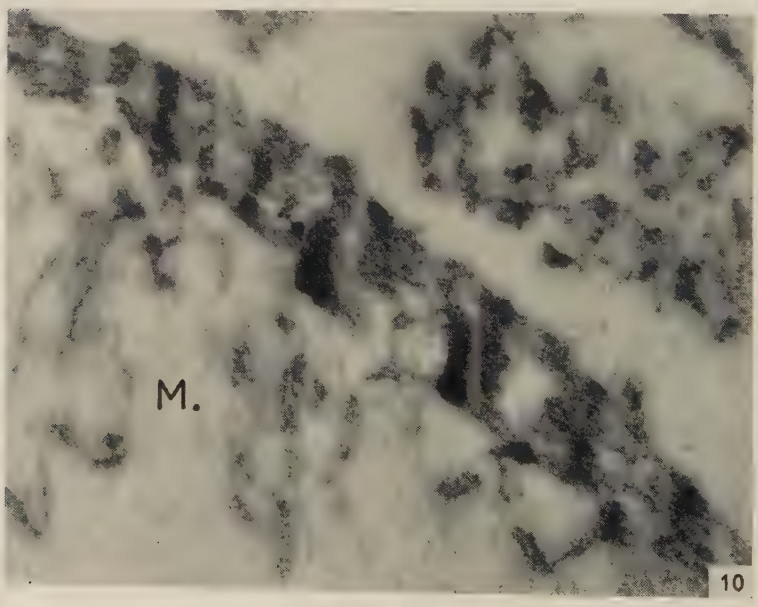
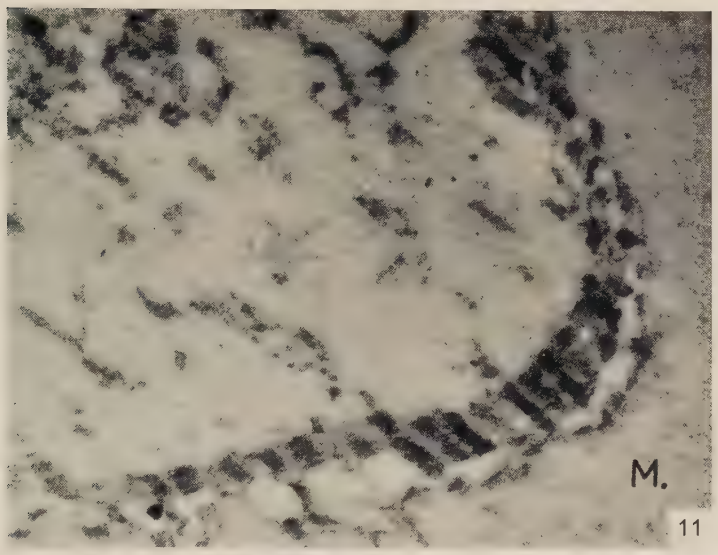


Fig. 9, 10 et 11. Micrographies électroniques de muscles striés du velum de *Limnocnida*, montrant la structure, la disposition et les points d'attaches des éléments musculaires. *M*, mésoglée. Grossissements : Fig. 9, 73.500 ×; Fig. 10, 106.000 ×; Fig. 11, 69.000 ×.



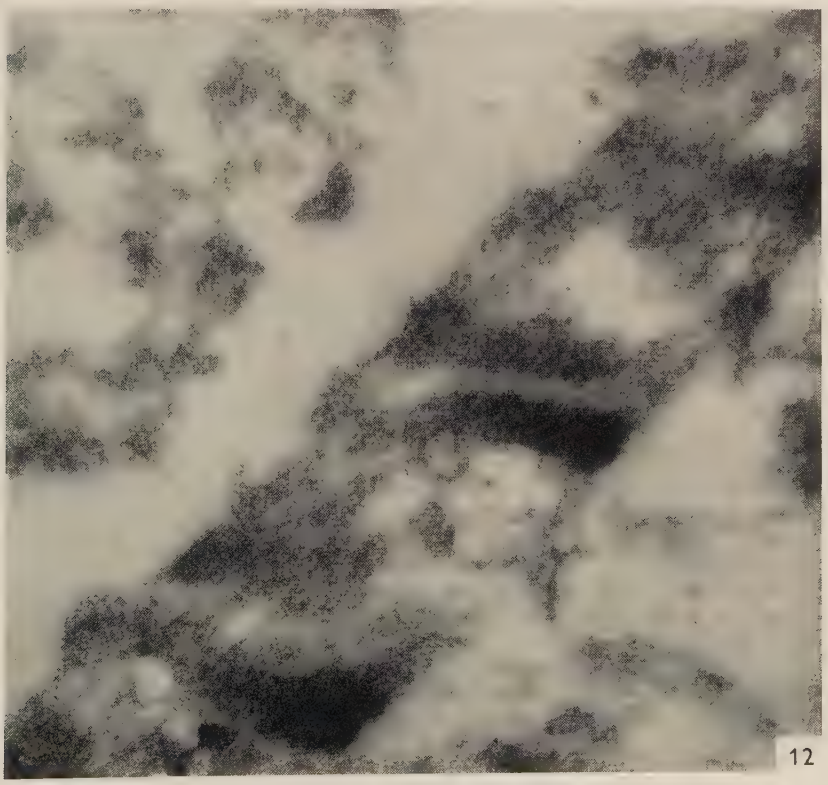


Fig. 12.—Portion de la Fig. 10 fortement agrandie. Grossissement 227.200 ×.

la paroi capsulaire des filaments internes ainsi que sur la génèse des nématocystes. La question de la structure de la paroi capsulaire a été longuement discutée [26]. Pour certains auteurs elle serait simple pour d'autres elle serait double, pour d'autres encore elle serait triple.

L'examen au microscope électronique permet de distinguer trois zones dans cette paroi. On observe une zone médiane épaisse (0,2 μ) d'aspect homogène et amorphe, limitée extérieurement et intérieurement par une pellicule granuleuse de texture hétérogène. (Fig. 14 et 15.)

La région externe pourrait correspondre à une condensation du cytoplasme de la cellule aux dépens de laquelle s'est différenciée la vésicule nématocystaire; la région granuleuse interne correspondrait peut-être aux limites de la vacuole nématocystaire. Il faut noter toutefois que l'observation de nématocystes dévaginés au microscope optique montre la présence d'une membrane intracapsulaire frippée distincte de la paroi et qui pourrait s'apparenter à la couche granuleuse interne. L'examen de coupes ultrafines de nématocystes dévaginés nous donnera la solution du problème.

La Fig. 15 nous montre une portion de nématocyste coupé longitudinalement; on distingue à droite la hampe (H) et le filament (F) coupés superficiellement. A l'intérieur de la hampe on remarque une série d'épines invaginées. A gauche de la figure on distingue quelques coupes transversales du filament (F). On note la présence d'épines.

La paroi capsulaire (P) de cette portion de nématocystes est recouverte intérieurement et extérieurement de granules; du côté interne on remarque à gauche une série de renforcements plus denses aux électrons.

Schulze [20] a signalé l'existence de grossissements localisés sous l'opercule chez les sténotèles de l'hydre. Il pourrait s'agir de structures identiques. En ce qui concerne la cnidogénèse, l'anneau urticant offre un matériel particulièrement intéressant, puisque c'est à son niveau que se forment les nématocystes. Nous pourrons donc espérer y trouver tous les stades de transition. De plus il n'existe ches *Limnocnida* qu'un seul type de nématocyste, des eurytèles hétérotriches microbasiques, toutes les figures observées se rapportent donc à la même évolution.

Nous ne montrerons ici que quelques figures illustrant l'aspect général du phénomène tel que nous pouvons l'interprèter à l'aide de nos premières observations au microscope électronique.

Les hypothèses développées ci-dessous doivent être considérées comme étant préliminaires. Ces recherches sont actuellement en cours et portent sur différents types de nématocystes tant au repos que dévaginés.

La Fig. 13 nous montre un fragment de l'anneau urticant, à gauche on

observe un nématoblaste au premier stade de son évolution. A l'intérieur de la cellule on distingue la vésicule nématoblastique (*V*) où la paroi capsulaire se différencie déjà ainsi que le noyau (*N*) très allongé qui lui est accolé.

La Fig. 14 correspond à un nématoblaste un peu plus âgé, la paroi capsulaire (*P*) est définitivement constituée, et l'intérieur de la vésicule est occupé par une vacuole (*V*) de structure hétérogène.

L'étude de nombreuses micrographies électroniques semble montrer que cette vacuole se condense en se transformant en un épais filament enroulé plusieurs fois sur lui-même au sein de la vésicule et entouré sur toute sa longueur par une gaine vacuolaire.

Les Fig. 16, 17 et 18 nous montrent des coupes transversales de tels stades, on y remarque les filaments très denses aux électrons et les limites de la vacuole. Ces figures illustrent également le fait que les sections du filament sont de diamètre variable (certaines portions correspondent peut-être à la hampe, d'autres portions correspondent au tube nématocystaire).

Primitivement homogène ce filament subit des modifications importantes. Il va s'évider et l'on voit des structures épineuses se différencier en son sein. Les Fig. 17 et 18 nous montrent diverses phases de la transformation du filament initialement compact en une structure creuse, tubulaire armée intérieurement d'épines.

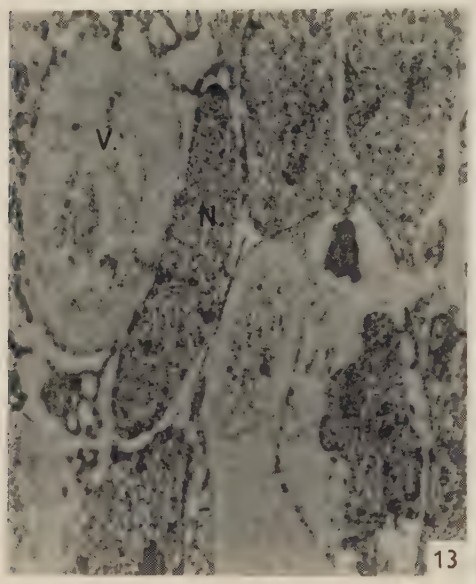
De nombreuses lacunes subsistent encore dans cet essai d'interprétation de la cnidogénèse, pour ne citer que la formation de l'opercule et la soudure des structures filamenteuses au niveau de la paroi capsulaire.

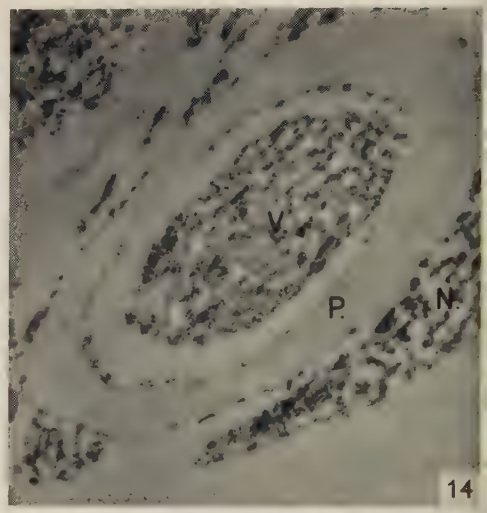
L'existence de structures épineuses à l'intérieur des filaments et de la hampe est toutefois clairement démontrée et nous semble un argument supplémentaire en faveur de l'hypothèse de la dévagination du filament nématocytaire.

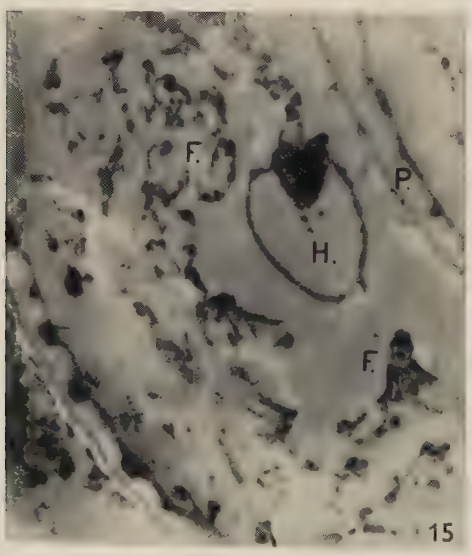
La formation de la hampe et du tube nématocytaire par différenciation sur place aux dépens d'un filament préalablement constitué nous permet d'exclure les différentes et souvent complexes théories invoquées pour expliquer la croissance des structures filamenteuses intra-capsulaires.

En dépit de leur caractère fragmentaire ces recherches nous permettent de confirmer des structures mises en évidence par la microscopie optique tout en apportant des précisions qui permettent éventuellement de mieux comprendre le fonctionnement de certains organites.

L'avenir nous permettra d'approfondir chacun des problèmes soulevés.







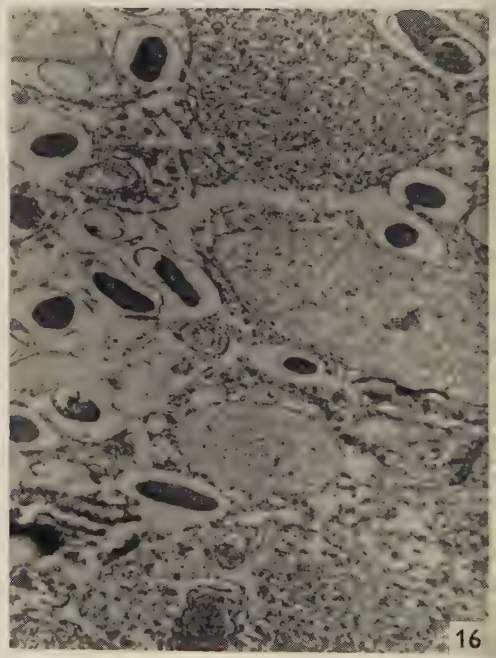
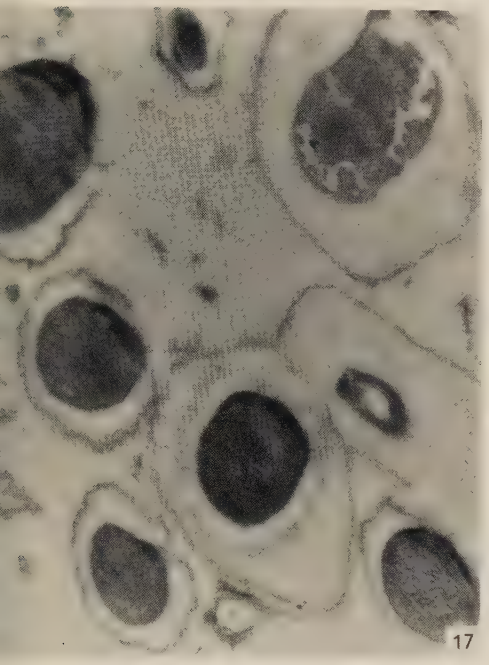


Fig. 13, 14, 16, 17 et 18.— Micrographies électroniques de coupes de fragments d'anneau urticant de *Limnocnida* montrant quelques aspects de la cnidogénèse. *N*, noyau; *P*, paroi du nématocyste; *V*, vésicule nématocytaire. Grossissements : Fig. 13, 28.000 × ; Fig. 14, 29.400 × ; Fig. 16, 19.600 × ; Fig. 17, 86.100 × ; Fig. 18, 86.100 × .

Fig. 15.—Coupe d'un nématocyste adulte de *Limnocnida* (Eurytèle microbasique hétérotriche). On distingue la paroi de la vésicule (*P*), le filament coupé longitudinalement et transversalement (*F*) ainsi que la hampe (*H*). Grossissement 30.800 × .



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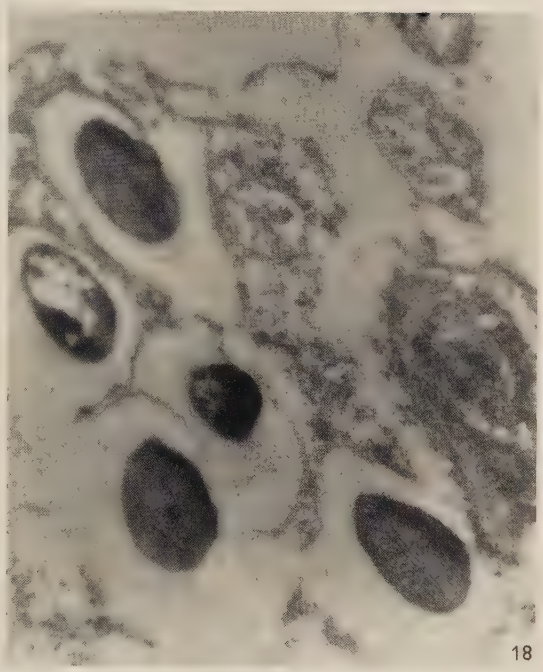

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RÉSUMÉ

L'histologie de différentes parties de *Limnocnida tanganyicae* (Limnoméduse) a été étudiée à l'aide du microscope électronique, en particulier l'anneau urticant, le canal circulaire, le velum et les tentacules ont été décrits.

Quelques hypothèses sont avancées : une interprétation de la cnidogénèse est donnée.

SUMMARY

The histology of different parts of *Limnocnida tanganyicae* (Limnomedusae) has been studied with the electron microscope: the nettle-ring, the marginal canal, the velum and the tentacles have been described. Some hypotheses have been proposed; an interpretation of the cnidogenesis has been given.

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Experimental Cell Research **13**, *545–552 (1957)*

THE CYTOLOGY OF THE IRRADIATED OVARY OF *DROSOPHILA MELANOGASTER*[1]

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Received May 7, 1957

Recent studies [2, 3] have been published which describe the cytology of the normal ovary of the adult fruit fly, *Drosophila melanogaster* and radiation-induced ovarian tumors in this insect. It is the purpose of this paper to describe other radiation-induced cytological changes in the ovary.

MATERIAL AND METHODS

Freshly eclosed female flies belonging to the Oregon-R, wild type strain were irradiated with 4000 r of Cobalt^{60} gamma rays ($\sim$100 r/second), and they were then placed with untreated males on medium for varying time intervals (generally 12 days). They were killed subsequently, and Feulgen whole mounts were made of their ovaries. Microscopic observations were made in normal bright field or phase contrast. In cases where the situation warranted, tracings were made of abnormal chambers using a Seibert Promi projection microscope, or photomicrographs were taken using Adox KB14 film in a Leica with a Micro-Ibso attachment. Approximately 500 ovaries were examined in both the control and the treated series.

RESULTS AND CONCLUSIONS

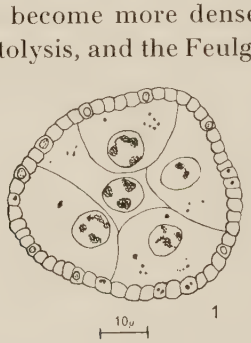
Early effects.—A cytological examination was made of the ovaries of flies killed 1, 2, and 6 days after irradiation. The ovaries from the 1- and 2-day series showed large numbers of Feulgen-positive granules distributed homogeneously throughout the cytoplasm of all cells (cf. Fig. 1). The majority of these granules have disappeared by the 6th day.

Late effects.—All the following information applies to the ovaries of flies killed 12 days after treatment.

Pycnosis.—Normally every ovariole of the 12-day-old *Drosophila melanogaster* contains oocytes in stages 1, 2, 3, 6, 7 and 14. The majority of ovarioles also contain stage 4 oocytes, and roughly half of the ovarioles also contain stage 8 oocytes. One stage 5 oocyte is found in every 5th ovariole. Oocytes

[1] Research supported by the U.S. Atomic Energy Commission (contract No. AT (11-1)-89, project 12) and the Graduate School of Northwestern University.

in stages 9 and 10 are found rarely (1 per ovary), and stages 11, 12 and 13 are absent. About 15 per cent of stage 8 chambers are pycnotic. In the irradiated ovary pycnotic chambers belonging to stages 1, 2, 3, 4, 6, 7, 9 and 10 have been observed in addition to stage 8. Degenerating nurse cell nuclei shrink and become more densely stained as a consequence. The cell walls undergo cytolysis, and the Feulgen-positive blobs representing the nurse nuclei



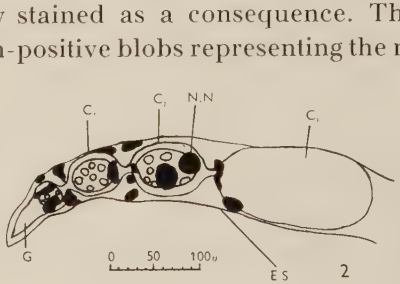


Fig. 1.—A camera lucida drawing of the Feulgen positive granules found distributed through the cytoplasm of a stage 4 chamber one day after irradiation.
Fig. 2.—An ovariole observed 12 days after irradiation. Nurse nuclei have ruptured from the posterior-most chamber (stage 8) and have passed into the anterior portion of the ovariole. *C*, chamber; *G*, germarium; *E.S.*, epithelial sheath; *N.N.*, nurse nucleus.

may rupture into the oocyte in the case of stage 8, 9, 10 or 11 chambers. Alternatively, the nurse nuclei may rupture the anterior follicular epithelium of the chamber and pass into the ovariole, where they become wedged between other chambers or the germarium and the epithelial sheath (cf. Fig. 2). Subsequent to the cytolysis of several chambers the ovariole itself may begin to degenerate. Such ovarioles are seen quite commonly (~ 1 per ovary), and they generally contain debris consisting of Feulgen positive blobs of various sizes which represent nurse and follicle cell nuclei (cf. Fig. 3).

Growth.—One of the commonest effects of irradiation is a general slowing down of oogenesis which in turn leads to an abnormal stage distribution. For example, the frequency with which chambers in stages 10, 11 and 12 occur is markedly increased (cf. Table I). Modified seriation represents are rarer type of abnormality. Normally adjacent chambers in any ovariole are never in the same stage. However, in irradiated ovarioles chamber sequences such as the following have been observed: 1, 3, 3, 3, 3, 10; 1, 3, 3, 3, 3, 8; 1, 2, 4, 5, 5, 5, 5, 5; and 2, 3, 4, 5, 6, 6, 7, 8, 12. One ovary was found which contained over fifty stage 14 oocytes. Apparently the egg-laying apparatus failed to function normally in this case.

Cell division.—A second common effect of irradiation is inhibition of cell division. Anaphase and metaphase chromosome figures are rarely found in

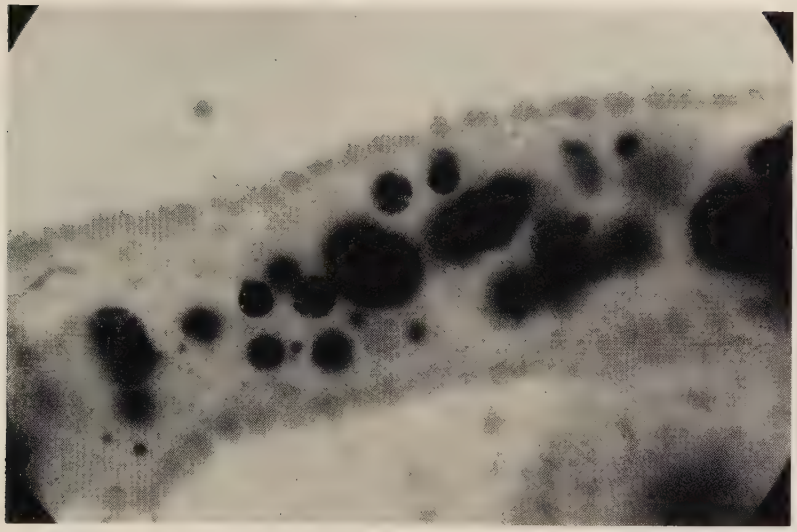


Fig. 3.—A photomicrograph of a degenerating ovariole.

TABLE I. *Morphological differences between control and irradiated ovaries.*

	Control	Treated
Germaria	large, sausage-shaped	small, blunt
Oogonial metaphases and anaphases	frequent	absent
Av. developing eggs/ovariole	6.97 ± 0.62 ($n = 30$)[a]	6.45 ± 0.75 ($n = 68$)[a]
Av. total ovarioles/female	25.06 ± 1.18 ($n = 17$)[b]	22.90 ± 1.02 ($n = 20$)[b]
Av. total developing eggs/female	175	148
Stage 10 oocytes/female	2.66 ± 0.74 ($n = 18$)	17.7 ± 1.03 ($n = 20$)
11	0	1.50 ± 0.32 ($n = 20$)
12	0.44 ± 0.20 ($n = 18$)	5.90 ± 0.70 ($n = 20$)
Frequency of ovarioles with x developing eggs		
$x = 6$	0.10	0.59
7	0.83	0.37
8	0.07	0.04
Total ovarioles	30	68

[a] These means are not significantly different, $P = 0.7$.
[b] These means are highly significantly different, $P = 0.009$.

irradiated germaria. Many of the Feulgen-positive blobs observed in the 1- and 2-day ovaries probably represent chromosome fragments. The number of developing eggs in treated ovaries is 85 per cent of the control. This is due to there being fewer ovarioles and fewer oocytes per ovariole (cf. Table I). Although radiation generally inhibits cell division, in specific rare instances it accelerates it. One example of this acceleration is the production of ovarian tumors [2]. A second is the production of chambers which contain cells which have undergone a supernumerary division. The two such eggs observed contained 23 nurse cells and the oocyte contained 2 nuclei (cf. Figs. 4, 5). Excess numbers of nurse cells (21–29) have been found in stage 7, 8, 9 and 10 chambers. These chambers all contained a single oocyte, however, and it is impossible to decide whether they resulted from fusions of two chambers or from a supernumerary division. The reverse situation, namely chambers with less than the normal cell number, have also been observed. In these cases the situation is always an extreme one. That is the chambers contain a few (1–4) very large nurse cells.

Cell migration.—Egg chambers pass down the ovariole as they develop. Irradiation appears to partially inhibit this process with the result that adjacent chambers often fuse together. In this respect irradiation has produced a phenocopy of the gene *comb-gap* [1]. The following classes of fusions have been observed: 2/3, 3/4, 4/5, 7/8 and 10/14. Fusions occur in the control series, but at a rate at least 50 times lower. Fusion almost always leads to a reversal of polarity of the anterior oocyte. Normally the most posterior of the 16 cells in a chamber differentiates into the oocyte. When two chambers fuse the cells of the posterior chamber behave normally. The oocyte of the anterior chamber, however, moves anteriorly and eventually becomes the most anterior cell of the chamber. The result is a chamber containing two groups of nurse cells which differ in size and stage and two oocytes—one at each pole (cf. Fig. 6). Often as the nurse cells grow the anterior oocyte will be crushed. The resulting chamber at a later stage may appear perfectly normal except for the abnormally high number of nurses. The chamber in Fig. 7 is probably the result of such a sequence of events.

Fig. 8 shows an interesting, but extremely rare abnormality observed in irradiated material. Subsequent to its determination the oocyte seems to have migrated to the center of the chamber. In spite of this, however, yolk is being laid down at the posterior pole as would normally occur in stage 8. This bizarre chamber demonstrates that the physical presence of the oocyte in the posterior pole of the stage 8 chamber is not required for the transfer of yolk to this region.

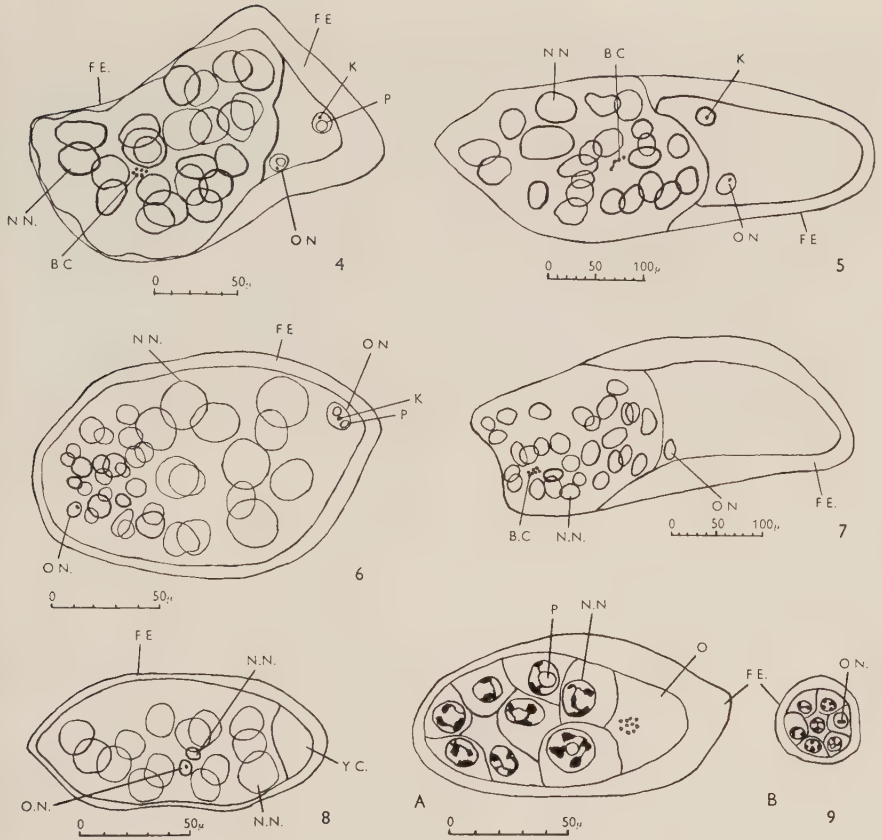


Fig. 4.—An outline drawing of an early stage 9 chamber (12-day series). It contains 23 nurse cells and 2 oocytes. *F.E.*, follicular epithelium; *O.N.*, oocyte nucleus; *P*, plasmosome; *B.C.*, border cells; *K*, karyosome.

Fig. 5.—An outline drawing of a late stage 10 chamber (12-day series). It contains 23 nurse cells and 2 oocytes.

Fig. 6.—An outline drawing of a composite chamber (12-day series) originating from the fusion of a stage 5 and a stage 6 chamber. It contains 37 nurse cells and 2 oocytes.

Fig. 7.—An outline drawing of a late stage 10 chamber (12-day series) which contains 29 nurse cells and 1 oocyte.

Fig. 8.—A stage 8 chamber (12-day series) containing a misplaced oocyte and a tiny nurse nucleus (*N.N.*). *Y.C.*, yolky cytoplasm.

Fig. 9.—(*A*) A stage 8 chamber (12-day series) with nurse nuclei arrested in differentiation at stage 4. The oocyte nucleus appears to have degenerated and only a series of vacuoles remain. (*B*) a normal stage 4 chamber. *O*, oocyte.

Differentiation.—Rare abnormalities have been observed which are interpreted as disturbances in differentiation. The first class of abnormality represents a homogeneous disturbance in that all nurse cells are similarly effected. What we find are chambers which can be classified as stage 8 on

the basis of the overall size, the follicular morphology, the size of the oocyte and that of the nurse nuclei. The nurse nuclei, however, are morphologically identical to stage 3 or 4. Thus nuclear differentiation has been arrested at stage 3 or 4 for the nurse cells, but this has not prevented further growth of these nuclei, nor has it prevented nurse cell cytoplasmic growth and yolk synthesis or the differentiation of the follicular epithelium. Fig. 9 illustrates a situation observed twice where the differentiation of nurse cells was arrested at stage 4. One case was observed where they stopped at stage 3.

The second class of abnormality represents a non-homogeneous disturbance in that not all the nurse cells of a chamber are affected similarly. The chamber represented in Fig. 8 shows an example of this phenomenon where the development of one of the nurse nuclei has been arrested. The reverse situation has also been observed. The case in point was a stage 3 chamber in which one of the 15 nurse nuclei had differentiated into a nucleus morphologically similar to a stage 6 nucleus but similar in size to a stage 3. A similar situation was observed once in a germarium. A stage 5 nurse nucleus was observed lying among oogonial cells.

Origin of border cells.—A large number of irradiated stage 9 and 10 chambers were observed in the hope of observing the migration of the border cells (cf. [3], p. 144). Various stages in the migration of these cells were observed (cf. Fig. 4, 5, 7). Apparently the cells originate early in stage 9 at the anterior pole of the egg and migrate posteriorly through the center of the nurse chamber until they reach the surface of the oocyte. Late in stage 10 the cells migrate back the way they came. After they have migrated about 100 micra into the nurse chamber they degenerate. Whether this degeneration also occurs in non-irradiated chambers is not known.

The chromosomes of the nurse cells.—We suggested in an earlier paper [3] that the chromosome number in the nurse cells remains constant and that as the chromosomes elongate they do not decrease in thickness because they simultaneously undergo a lateral replication and therefore become polytene. We observed in one stage 8 chamber a nurse nucleus which showed exceptional chromosomal detail (cf. Fig. 10). Normally the nurse nucleus at

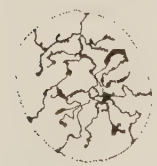


Fig. 10.—A camera lucida drawing of a stage 8 nurse cell nucleus which shows exceptional chromosomal detail.

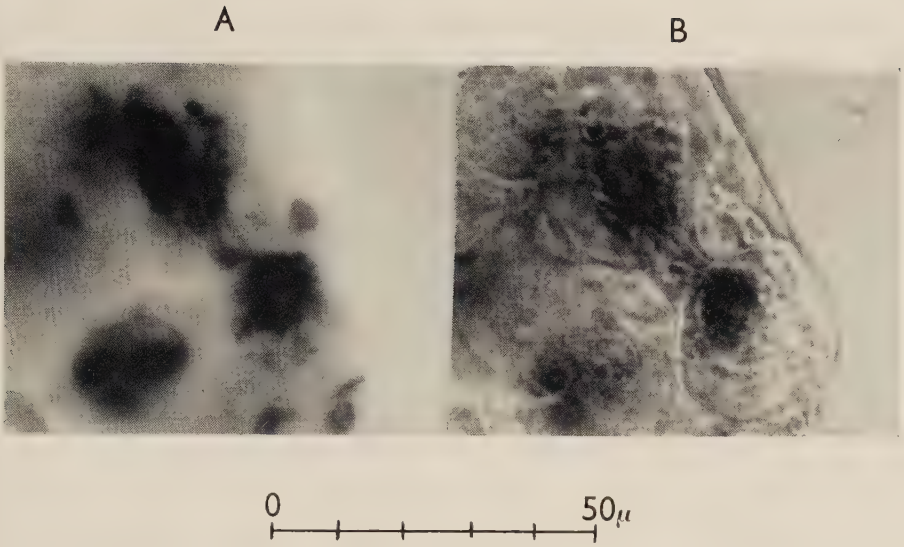


Fig. 11.—A photomicrograph of cytokinesis in a stage 9 nurse chamber. Note the cleavage furrow which is pinching the chromatin into two unequal masses. *A*, Bright field. *B*, Phase contrast.

stage 8 is completely filled with chromosomal material and therefore looks amorphous. However, the nucleus in question was relatively gigantic and the chromosomes within were clearly visible. A given chromosome segment shows marked variations in circumference which appear to result from variations in coiling. Certain regions show what appears to be a series of bands, but it is impossible to rule out the possibility that these may represent a compact series of gyres. Also in the irradiated material an instance was observed of cytokinesis in a stage 9 nurse chamber. Fig. 11 shows the nurse cell nucleus in question as it is pinched in two by the cleavage furrow.

DISCUSSION

The limited state of our present knowledge makes it impossible to make any valid statements as to the specific mechanisms involved in producing the abnormalities observed. About all one can say is that current studies in developmental genetics have led to the concept that the developmental activities of cells (growth, differentiation, migration, and controlled division) are the result of the interactions of various portions of the genome of given cells with specific molecules arranged in specific gene controlled cytoplasmic patterns. Perhaps the abnormalities observed result from radiation-induced

inactiviation or loss of various portions of the genetic machinery essential to such developmental sequences. For such an injured cell and its progeny normal developmental behavior is impossible, and depending on the specific genes removed from action there follows a specific type of abnormal behavior.

SUMMARY

A cytological study was made of the ovaries of 12-day-old *Drosophila melanogaster* given 4000 r Co^{60} gamma rays shortly after emergence. Many severely damaged oocytes underwent pycnotic degeneration. The number of developing eggs was reduced to 85 per cent the control value. Many of the surviving oocytes showed abnormalities which are ascribed to radiation induced disturbances of cell growth, division, migration and differentiation.

The author is grateful for the conscientious assistance performed by Mr. Jack Samuels. Thanks are also due to Dr. Malcolm Dole for providing the irradiation facilities.

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CULTIVATION AND BEHAVIOR *IN VITRO* OF THE NORMAL MAMMARY EPITHELIUM OF THE ADULT MOUSE

II. OBSERVATIONS ON THE SECRETORY ACTIVITY[1]

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Received May 17, 1957

We have recently described a method for the cultivation of the normal mammary epithelium of the adult mouse [9]. In accord with Grobstein's findings [7] it was then observed that fibroblasts, whenever present, far from inhibiting the growth of the epithelium, oriented it and to some extent stimulated its differentiation. Tubes and alveoli formed in the midst of the connective tissue, eventually displaying signs of functional activity by becoming distended with a more or less granular, eosinophilic coagulum.

In such a microscopic reproduction of the mammary gland, physiological mechanisms similar to those observed *in vivo* might be expected. Most striking however, was the extremely small number of fatty inclusions present in the epithelial cells; very few were released into the lumina of the alveoli. This being in direct opposition to the classical conception of milk-fat elaboration in the epithelium, such a discrepancy appeared worthy of investigation. We shall present in this report some preliminary results.

MATERIAL AND METHODS

The dissociation of the mammary gland with collagenase K 264 as we described it, depends on two main factors: time and agitation. Variations in length of time and intensity of agitation result in several degrees of dissociation, thus offering an opportunity to isolate the different cellular components of the organ. For instance, simple contact of mammary fragments with the collagenase solution for one hour at 37°C loosens the cells well enough to favor an epithelial migration. Further moderate agitation is usually necessary to bring about the mechanical separation of the adipose tissue from the glandular stroma and the epithelium. A longer period of treatment (1½ hours) has injurious effects on the connective tissue which may remain attached to the epithelium; almost pure epithelial cultures can then be

[1] This investigation was supported by a research grant (C 2520) from the National Institutes of Health, Public Health Service.

obtained. Such variations have been extremely useful in studying the fat elaboration in mammary glands taken from C 57 mice at different stages of pregnancy.

A *Palo* wrist-shaking type of agitator regulated at 120 rpm. is used. Since glass beads do not seem to increase significantly the speed of dissociation they have been eliminated.

The Maximow double coverslip method is employed. The culture medium, composed of one part human placental serum to two parts Simms' saline solution supplies all hormonal and nutritional requirements of the epithelium. The culture coverslips are first coated with a thin film of chicken plasma in the following way: a few drops of plasma are placed on the bottom of a Petri dish. With forceps, one side of the coverslip is gradually lowered on to the plasma until its entire surface is covered. The excess plasma is drained by holding the coverslip vertically, its edge touching sterile filter paper. One drop of the tissue suspension in the culture medium homogenized by pipetting is then distributed on each coverslip and the excess medium withdrawn. A sufficient amount of tissue extract is carried by the fragments themselves to insure a quick and firm setting on the coated glass surface. No embryo extract is used.

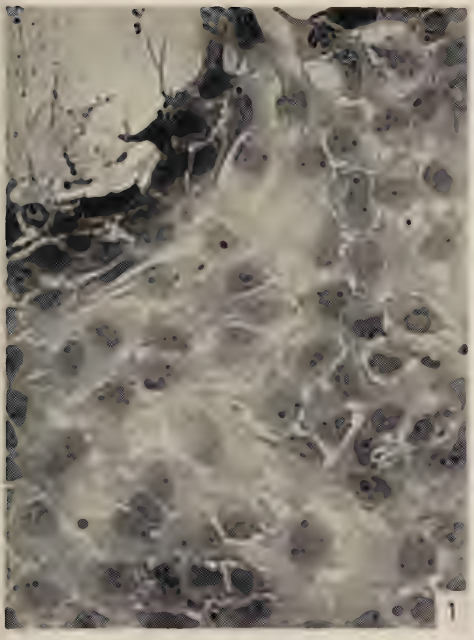
For fat demonstration, the cultures were fixed for 15 minutes in Palade's 1 per cent isotonic buffered solution of osmium tetroxide [6], then routinely stained by the Van Gieson method. Some cultures were also fixed with vapors from a 2 per cent aqueous osmium solution (half an hour), and still others were stained with a 1.5 per cent alcoholic solution of Sudan black or a 1 per cent aqueous solution of Nile blue sulfate (*Lorrain–Smith* test) after one hour's fixation in 10 per cent Locke-formalin. Finally a few observations were made directly on living cultures by phase-contrast microscopy.

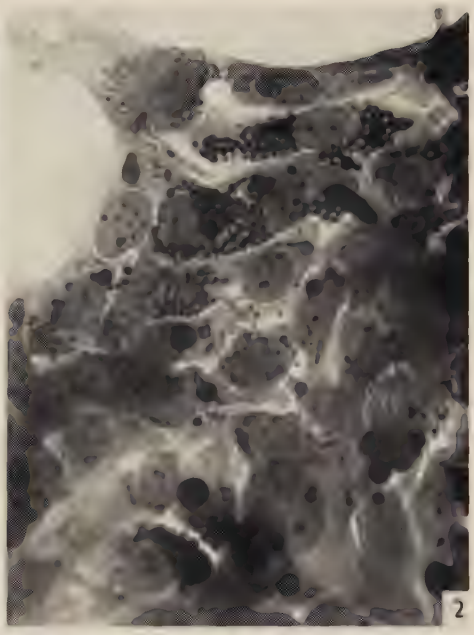
RESULTS

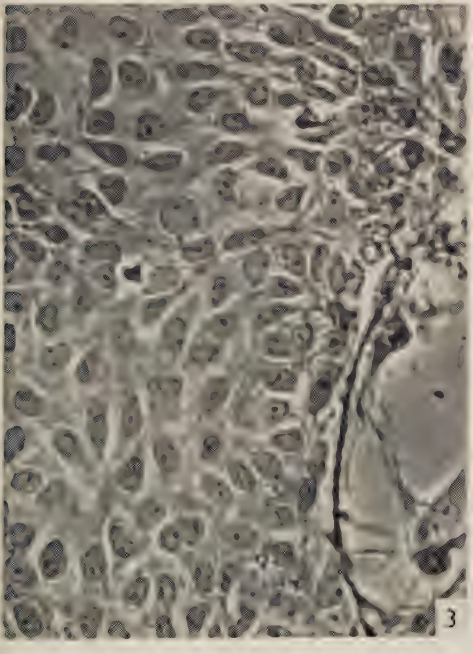
Mammary Glands at Middle Pregnancy

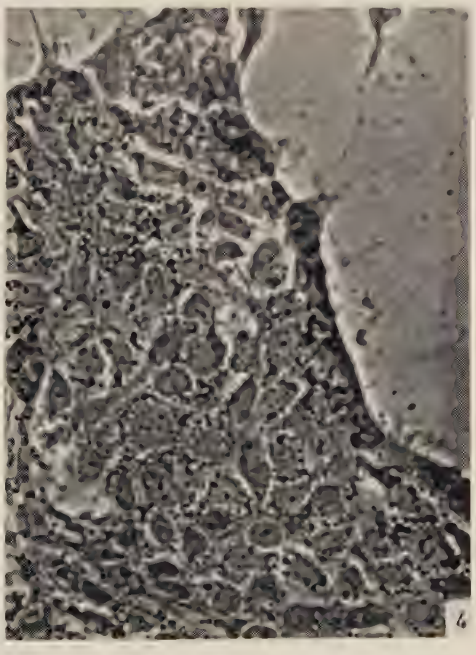
Total dissociation.—After prolonged treatment with collagenase (1 ½ hours agitation at 37°C), cultures of mammary glands taken at mid-pregnancy often give fairly pure epithelial colonies. They appear as cellular islands, spreading after 48 hours or so in monolayers of large cells irregular in shape and more or less loosely connected with one another. Their nuclei are relatively big and round, oval or sometimes bean-shaped; the cytoplasm is clear, extremely thin, and totally agranular. No lipid droplets can be observed either in the living cultures or after fixation in a buffered osmium solution (Fig. 1).

Fig. 1.—Mammary epithelium, mid-pregnancy, 8 days' cultivation; glandular stroma eliminated. Fixed in buffered osmium tetroxide and stained by Van Gieson's method. No fat inclusions. × 340.
Fig. 2.—Mammary epithelium, mid-pregnancy, 8 days' cultivation; glandular stroma retained: Osmium fixation; Van Gieson's stain. Numerous fatty inclusions. × 340.
Figs. 3 and 4.—Mammary epithelium, mid-pregnancy, 2 days' cultivation. Both preparations originate from the same mouse. In Fig. 3 the glandular stroma is totally eliminated and the cultures are free of fat inclusions. In Fig. 4 the stroma is retained and fatty inclusions are present. Sudan black stain. × 630.









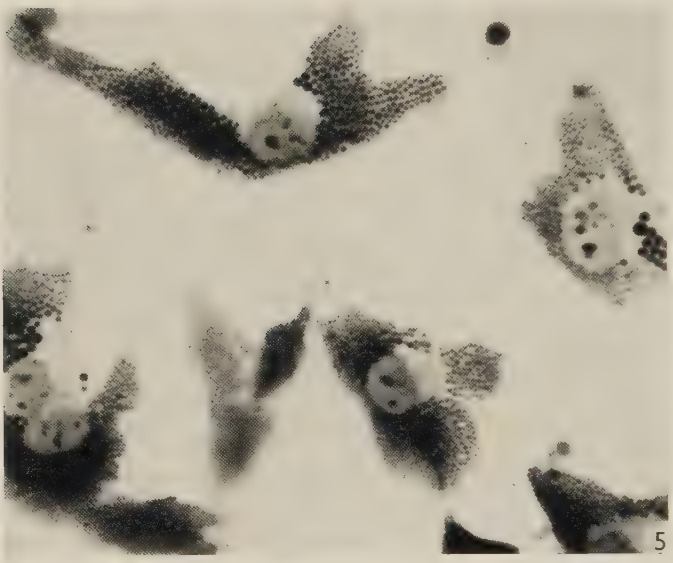


Fig. 5.—Large wandering adipose cells in the glandular stroma. Fixation by osmic vapor, Van Gieson stain. ×340.

At this stage of pregnancy, histological preparations show that the cytoplasm of the epithelium is already quite rich in fatty materials. Their absence in the cultures could mean that collagenase has favored a thorough extraction of the fat. In that case, if the epithelium is the actual site of lipid formation, fat droplets should reappear soon in the cytoplasm of the cells cultivated in an adequate medium. However, maintenance of the cultures during 14 days, and therefore beyond the term of the pregnancy period, failed to give evidence of new lipid formation.

Partial dissociation.—By contrast, if a similar mammary gland is only partially dissociated by the collagenase (standing 1 hour in the enzymatic solution at 37°C), the cultivated fragments give epithelial sheets with a multitude of fatty inclusions (Fig. 2). The presence of the glandular stroma delays for a few hours the migration of the epithelium while many other elements such as fibroblasts, large lymphocytes, macrophages, all more or less rich in fat, invade the medium. Numerous giant ameboid cells tightly packed with fat droplets and gliding conspicuously on the surface of the epithelium are particularly noticeable (Fig. 5). The explant itself is still surrounded by many fat globules incompletely loosened by the enzymatic action.

These results suggest that the lipids found in the epithelial cells come from the surrounding stroma. In a new set of experiments we repeated the same procedure on mammary glands taken from *one single mouse* (C57) at mid-

pregnancy. The organs are divided into two sets one of which is submitted to a complete, and the other to a partial dissociation by collagenase. Observation of the cultures after 48 hours confirms the above results: whereas the total dissociation yields cultures of pure epithelium free of lipid droplets (Fig. 3), the partial treatment, retaining most of the stroma of the gland, gives sheets of epithelium extremely rich in fatty inclusions (Fig. 4). Maintenance of the cultures over 30 days does not change the picture markedly, but the epithelium from a partially treated organ gradually loses its lipid droplets; the large ameboid fatty cells so numerous at the beginning of cultivation decrease rapidly in number while the globules of neutral fat retained in the stroma of the explant appear to remain totally inert.

Since both sets of cultures originated from the same animal and are maintained in the same conditions with identical media it appears highly probable that the surrounding stroma and particularly the large lipoid cells, play an important role in the processing of the fats found in the epithelium.

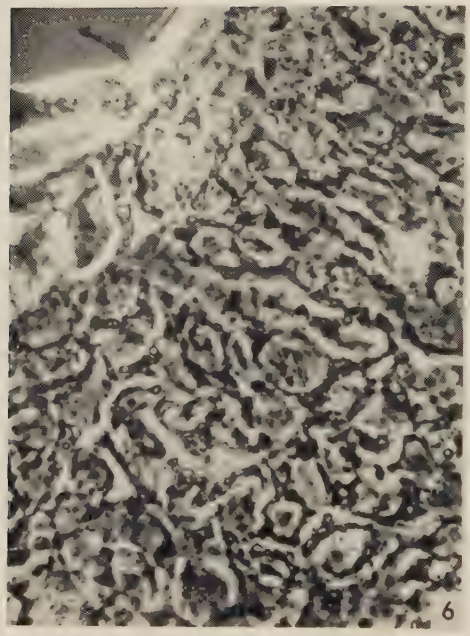
Mammary Glands at the End of Pregnancy

The mammary glands of a mouse taken a few hours before parturition, are fully developed and show all the characteristics of a secreting organ. If a complete separation by collagenase with vigorous shaking is done, we have, after several washings, cultures of epithelium displaying very many fat inclusions (Fig. 6). These inclusions are retained in the migrating cells for several days, then liberated after about a week either by the degeneration and disintegration of the cells or by other mechanical processes. After more prolonged cultivation, new epithelial cells appear with a less granular, clearer cytoplasm (Fig. 7).

Thus it would seem that the fat found in the epithelial cells at the beginning of cultivation has been stored by them prior to their *in vitro* life. Once isolated from the glandular stroma the epithelium seems incapable of generating new lipids.

Influence of Glandular Lipids

Whenever a mammary gland is dissociated by collagenase, a considerable amount of fatty substance is separated from the tissue. If the epithelium receives its fat from the surrounding stroma in the form of diffusible fatty acids, it is logical to expect their presence in the extracted material and a reappearance of fatty inclusions *in vitro* when these specific fatty acids are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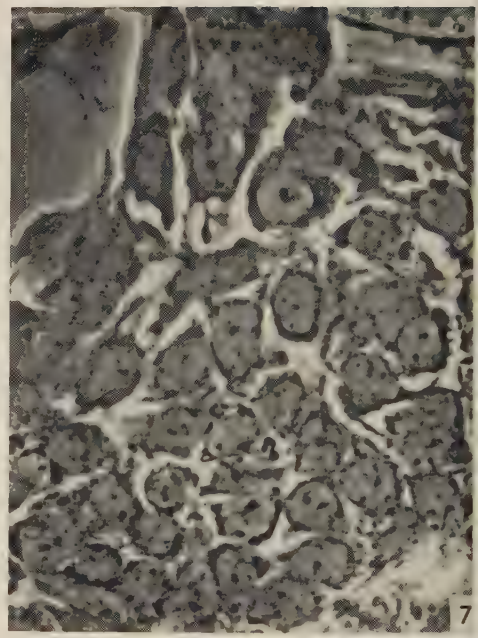
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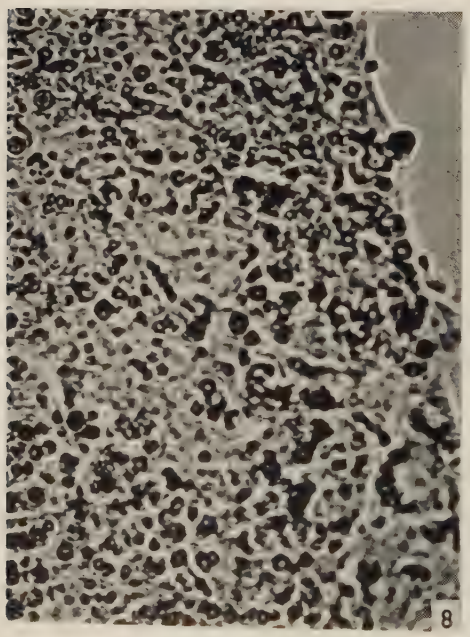
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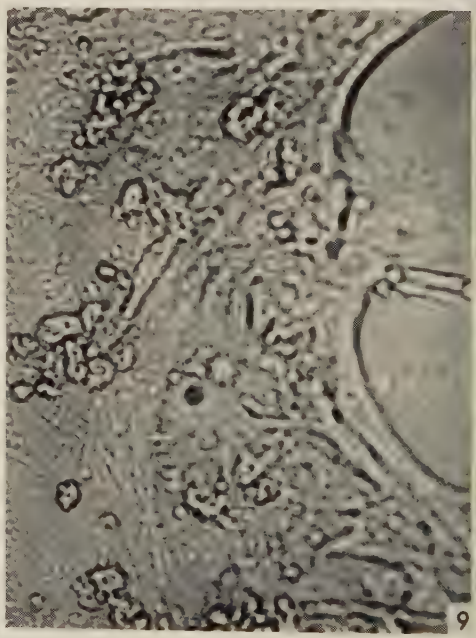
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added to the culture medium. We therefore collected the fatty material resulting from the dissociation of the gland and emulsified it finely in human placental serum at 37°C for one hour. This serum, after centrifugation, was used to make up the culture medium in the same concentration (1/3) as was formerly employed for the untreated serum.

After 4 days' cultivation, fat droplets began to accumulate in the epithelium while controls made with normal serum remained perfectly clear (Figs. 8, 9).

DISCUSSION

The experimental evidence just presented suggests that the mammary epithelium does not play a major part in the synthesis of fats but rather has an important role in their distribution and recombination.

Even at the dilutions used, the content of fat precursors in the placental serum is sufficient to enable cells specialized in fat production to exert their function. In undiluted serum even unspecialized cells such as ordinary fibroblasts can display a rather large amount of fat inclusions while adipose tissue can elaborate fatty materials from entirely synthetic media supplemented with insulin [14].

Other authors, however, have connected the activity of the adipose tissue in the mammary gland with milk-fat production. As early as 1922, Benoit [1, 2] called attention to giant wandering cells, extremely rich in fat, which in the lactating mammary gland of the mouse glide on the external epithelial surface of the acini. These giant cells which he named "lipopexic cells" would assume a fundamental role in the absorption and synthesis of the specific fatty materials of the milk. Benoit did not observe a direct passage of fat droplets into the epithelium and therefore suggested that fat was going through the basal membrane of the acini in a state of chemical dissociation; whereas the epithelium itself would be specialized for protein synthesis.

Fig. 6.—Mammary epithelium at the end of pregnancy, 5 days' cultivation; stroma totally eliminated. The epithelium is extremely granular and shows numerous fatty inclusions. Living culture. ×630.

Fig. 7.—Same epithelium as in Fig. 6, after 12 days' cultivation. The fat has been eliminated. Large nuclei are surrounded by a small amount of cytoplasm. No production of new fat globules. Living culture. ×630.

Fig. 8.—Mammary epithelium, mid-pregnancy, grown in serum containing an emulsion of fat from the glandular stroma. The fatty inclusions are again numerous. Living roller-tube culture, 8 days old. Fat introduced into the medium 4 days before. × 540.

Fig. 9.—Epithelium from the same mouse, control. Living roller-tube culture, 8 days old. ×540.

Benoit's hypothesis, mainly based on morphological observations, has recently found some experimental support. Using a ribonuclease, Vendrely [16] demonstrated that the mitochondria of the mammary epithelium contained ribonucleic acid whereas the mitochondria of the adipose cells had none. This author linked the presence of RNA to protein synthesis; fat on the contrary, would be elaborated by the mitochondria without RNA.

Our own tissue culture experiments afford more evidence in support of Benoit's view. In particular, they show that although the epithelium does not synthesize fat, a close cooperation exists in that respect with the glandular stroma at the lactation period. This stroma, which is quite complex, is difficult to analyze histologically, but we have observed that pure connective tissue, though necessary for organization of the epithelium, is not responsible for fat production. The adipose tissue therefore appears as specifically distinct from the connective tissue. Such a distinction, most significantly evidenced in tissue cultures of liposarcomas by Murray and Stout [10], has also been made by Wells [17], Rasmussen [12], and more recently by Sidman [14]. On the other hand, it is known that as pregnancy progresses, the mammary glands undergo a series of modifications in which both epithelium and adipose tissue, possibly under hormonal influences, are deeply involved: the fat embedding the epithelium becomes more fluid [8] while the alveolar epithelium develops extensively.

The role played at this stage by the adipose tissue and the epithelium is sharply outlined in tissue culture: the activity of the adipose tissue is expressed by the appearance of a great many giant ameboid cells filled with a multitude of refringent droplets suspended in an areolar cytoplasm. Nile blue sulfate stains seem to indicate that these lipid droplets are of a different nature from the neighboring neutral fat globules although both take equally well Sudan black or osmium tetroxide. We assume that the adipose cells have the specific function of breaking down the long chain fatty acids of neutral fat to release smaller diffusible molecules in the intercellular fluid. This view is most likely since the presence in the adipose tissue of diastases, phosphatases, lipases, and dehydrogenases has been reported [18]. In particular, the presence of lipolytic enzymes such as lipases has been noted by several investigators [5, 6]. It would be interesting to determine the lipase activity in the adipose tissue of the mammary gland and its variations in relation to the different stages of gestation.

We have seen that the epithelium does not produce any fatty material in the absence of adipose tissue, but we have shown that the fat collected after dissociation of the gland, when emulsified in serum, is readily absorbable.

This would agree with the results reported by Bloor [4], that when hydrolysis of fat has proceeded to glycerol and fatty acids, the absorption of these intermediates occurs easily. It could be objected that the lipid inclusions seen in the epithelial cells may result from an excess of free oleic acid in the medium. Jacquez and Barry [8] observed in tissue cultures the toxicity of this particular fatty acid which exists in quite a high percentage in milk fat. This possibility is unlikely however, in view of the fact that fibroblasts, growing in symbiosis with the epithelium, remained reasonably free from lipid inclusions. On the other hand, Biesele and Goldhaber [3] have shown that massive fatty accumulation is not necessarily a sign of unhealthy condition. After entering the epithelial cytoplasm, the fatty materials are probably associated with proteins or recombined into phospholipids and butter-fat.

CONCLUSIONS

It appears that cultures of mammary epithelium can organize and eventually resume a physiological function when associated with connective tissue. Milk-fat, however, is not produced under these circumstances. The following experimental evidence indicates the influence of adipose tissue upon the accumulation of fat in the epithelium:

(1) Fairly pure cultures of mammary epithelium at mid-pregnancy are and remain free of fatty inclusions, even when the cultures are maintained for periods of time beyond the regular term of pregnancy in the mouse.

(2) Retention of the glandular stroma in the cultures after an incomplete dissociation by collagenase, gives epithelial sheets very rich in fatty droplets. Identical results obtained with cultures of mammary epithelium from one single mouse suggest that the fat found in the epithelium originates in the stroma.

(3) Pure cultures of epithelium at the end of pregnancy are extremely rich in fat but lose it almost completely after 15 days' cultivation. This would confirm that the mammary epithelium does not elaborate fat by itself, but is able to receive and store it, at least for some time.

This evidence suggests the following hypothesis: neutral fat, hydrolyzed in the large lipoid cells of the adipose tissue, possibly under the influence of lipolytic enzymes, is released in the interstitial fluid whence it is absorbed by the epithelium. In turn, the epithelium associates the lipids absorbed with proteins or recombines them into the characteristic milk-fat molecules. Thus, the adipose tissue and the epithelium of the mammary gland would have distinct but complementary functions for the final elaboration of milk.

SUMMARY

Cultures of mammary epithelium of the adult mouse can grow and organize when associated with plain connective tissue. Even though signs of secretion can be observed in such organized units, the absence of lipidic inclusions indicates that milk-fat is not primarily elaborated in the epithelium. Experimental evidence is given showing that milk-fat production is possibly under the specific control of the adipose tissue.

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INTRACELLUAR STRUCTURES IN *STRIGOMONAS ONCOPELTI*

I. CYTOPLASMIC STRUCTURES CONTAINING RIBONUCLEOPROTEIN

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In 1913 Wenyen [34] described rod-like structures in the cytoplasm of the flagellate *Herpetomonas muscae*; the structures showed a tendency to bipolar staining and were always located at the posterior end of the flagellates. Wenyen at first thought they were composed of chromatin material extruded from the nucleus, but he later suggested that they were bacteria ingested by the flagellates as food. Ten years later Becker [1] examined the same strain of *Herpetomonas*; he stated that these flagellates were osmotrophes and expressed the view that the rod-like particles were nuclear fragments which occurred only in degenerating organisms. No further account of these structures has been found in the literature. Recently [24] similar particles were observed in the cytoplasm of the trypanosomid flagellate *Strigomonas* [*Herpetomonas*] *oncopelti*; in the present paper a more detailed description of these particles will be given together with evidence which suggests that they are self-reproducing organelles composed largely of ribonucleoprotein [RNP].

METHODS

Organism and growth media.—The strain of *Strigomonas oncopelti* used was obtained from Dr. Muriel Robertson, F.R.S., Lister Institute, London. Stock cultures were maintained in 5 ml volumes of 3 per cent (w/v) Evans Bacteriological Peptone containing 0.5 per cent (w/v) glucose and 0.5 per cent (w/v) sodium chloride, at an initial pH value of 7.4. The medium was sterilized by autoclaving at 15 lb/sq.in. for 20 min. Cultures were incubated at 25°C and subcultured every other day. Larger batches of organisms were grown in 50 or 500 ml of the above medium contained in either 150 ml or 1 litre flasks; a 10 per cent inoculum from a 15-hour culture was used in all cases. For some experiments organisms were grown in a chemically defined medium containing glucose, methionine, thiamine, nicotinamide, *p*-aminobenzoic acid, salts and trace metals as previously described [23].

Cytochemical techniques.—'Smears' of organisms were prepared on microscope slides, dried in air and fixed by immersion in absolute methanol for 10 min. Fixed

preparations were then stained by one of the techniques listed in Table I. Enzymes used were: crystalline, protease-free, ribonuclease (RNase) and crystalline deoxyribonuclease (DNase) supplied by Sigma Chemical Co., U.S.A. Enzyme solutions (0.1 per cent w/v) were prepared in glass distilled water, containing in the case of DNase, 0.075 *M* $MgSO_4$. Preparations were incubated in enzyme solutions at 37°C for varying periods of time; in each experiment a control preparation was incubated in glass distilled water alone.

Fractionation of cells.—Organisms were harvested by centrifugation, washed once with 0.85 per cent saline and then resuspended in 2.5 *M* sucrose to give a final cell density of ca. 5×10^8 10^9 cells/ml. All sucrose solutions used for cell fractionation contained 0.3 per cent sodium citrate. 10 ml samples of cell suspensions were mixed with an equal volume of small glass beads (Chance Bros. Ballotini, No. 12) and shaken in a Mickle [22] disintegrator for 4 min. This resulted in the breakage of more than 90 per cent of the cells. After removal of unbroken cells by centrifugation at 1500 × g for 10 min. the preparation was centrifuged at 6000 × g for 10 min., the resultant pellet was washed once with 2.5 *M* sucrose and finally suspended in 2 ml of 2.5 *M* sucrose before layering over a medium of graded sucrose concentration and centrifuging for 10 min. at 3000 × g. The medium of graded sucrose concentration was prepared in a 15 ml centrifuge tube, a bottom layer of 3 ml of 15 *M* sucrose was used and over this 2 ml layers of sucrose solutions were carefully introduced with a bent-tipped pipette; concentrations used were as follows: 13 *M*, 9 *M*, 5 *M*, and 2.5 *M*. After centrifuging, layers were removed with a bent-tipped pipette.

Analysis of cell fractions.—Cell fractions to be analysed were precipitated with perchloric acid (final concentration of 0.2 *N*). After standing for 1 hr at 5°C the precipitates were centrifuged down, washed twice with cold 0.2 *N* perchloric acid and extracted three times with 75 per cent ethanol-ether (1:1 w/v), to remove lipid. The residue was then extracted three times with 0.5 *N* perchloric acid at 70°C for 30 min.; 90–96 per cent of the total nucleic acid was extracted in the first two treatments and no further extraction took place after the third treatment. Samples of the combined 0.5 *N* perchloric acid extracts were taken for the determination of total nucleic acid by measurement of absorption at 258 mμ in a Beckman Spectrophotometer (Model DU), for the determination of deoxyribose by the diphenylamine method of Burton [5] and for the determination of total phosphorus, after digestion with sulphuric and nitric acid, by the method of Fiske and Subbarow [13]. The total nitrogen content of the residue remaining after extraction with perchloric acid was determined colorimetrically with Nessler's reagent as described by Johnson [18], after digestion by the Kjeldahl method under the conditions described by Chibnall, Rees and Williams [11].

Preparation of specimens for electron microscopy. Intact cells were fixed in 1 per cent buffered osmium tetroxide (pH 7.5) as described by Porter and Kallmann [28] and Palade [26]. After fixation for periods ranging from 30–90 min. preparations were dehydrated by passing through a series of graded alcohols before being embedded in a mixture of 9 parts butyl- to 1 part methyl-acrylate; the embedding medium was polymerized in an oven at 45°C. Sections were cut and examined in the Siemens 'Elmiskop' electron microscope operating at 80 Kv; instrumental magnifications of 5000×, 10,000× and 20,000× were used. It was found that a fixation time of

TABLE I. *Summary of cytochemical studies.*

Stain	Reference to method used	Staining reaction of bipolar bodies
Iodine	—	-ve
P.A.S.	Hotchkiss [16]	-ve
Bismark Brown	Leach [19]	-ve
Sudan Black	Burdon [4]	-ve
Toluidine Blue	Lindegren [20]	euchromatic
Neisser's stain	—	-ve
Feulgen reaction	Tomasi [31]	-ve
Methyl Green	White [35]	-ve
Pyronin G	White [35]	red
May-Grünwald and Giemsa	Jacobson and Webb [17]	blue

30 min. was the most satisfactory under the conditions used, but alternative methods may prove to be necessary for the further study of fine structure [14].

Isolated bipolar bodies, suspended in 2.5 *M* sucrose, were prepared for electron microscopy by fixation in either 1 per cent formalin or 1 per cent buffered osmium tetroxide. Fixed preparations were washed free of sucrose with distilled water and microdrops of suspensions in water were placed on specimen grids covered with nitrocellulose film. The specimens were shadowed by evaporating uranium onto them at an angle of 50°.

RESULTS

Phase contrast microscopy.—Bipolar rod-like particles were first observed in broken cell preparations of *Strigomonas oncopelti* by phase contrast microscopy [24]; these particles were only found when cells were broken in the presence of 2.5 *M* sucrose, breakage in isotonic saline or in distilled water resulted in a loss of these structures. No intracellular structures could be seen when intact organisms were examined under the phase contrast microscope; if, however, a suspension of organisms in 2.5 *M* sucrose was gently squashed between a cover glass and microscope slide bipolar structures could be clearly seen in many cells in addition to a nucleus and kinetoplast (Fig. 1).

Cytochemical studies.—The results of a series of cytochemical tests, summarized in Table I, suggest that the bipolar bodies observed in unfixed preparations by phase contrast microscopy may contain ribonucleic acid [RNA]. These structures were stained blue by May–Grünwald–Giemsa dyes and red by pyronin G; they were not stained by Feulgen's method or by methyl green.

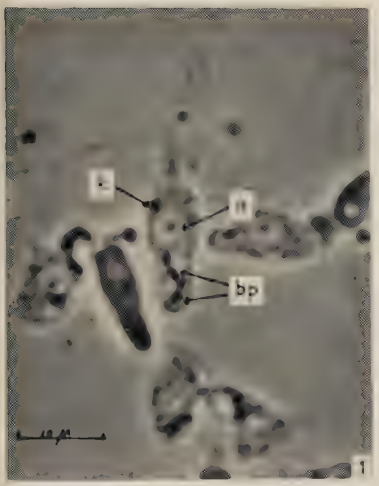
Treatment with nucleases.—Further evidence for the association of RNA

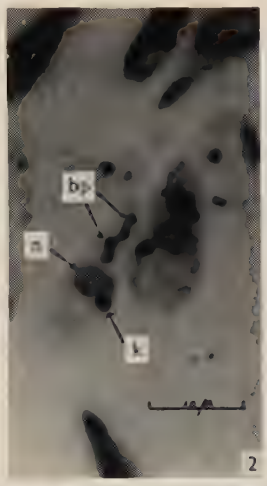
with these structures was obtained by treatment of fixed preparations of *Strigomonas* with nucleases. After 45–60 min. incubation at 37°C with RNase all material which stained blue with May-Grünwald-Giemsa dyes had been removed from the cells (Figs. 2, 3 and 4); nuclei and kinetoplasts were unaffected by this treatment and were stained red. Control preparations incubated for the same period of time in glass distilled water showed no appreciable loss in basophilia. In agreement with these results a complete loss of pyronin stainable material resulted from treatment with RNase whereas the methyl green-positive material in the cells was unaffected. No alteration in the staining characteristics of the bipolar bodies resulted from incubation of fixed preparations of organisms with DNase for 60 min. at 37°C.

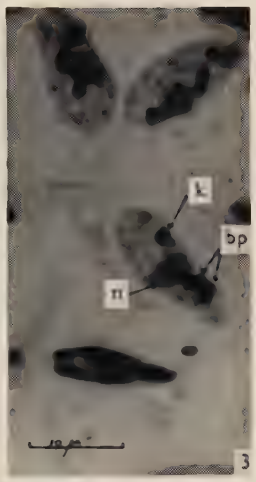
Inhibition of ribonuclease activity.—To confirm that the loss in basophilia resulting from RNase treatment was due to enzymic activity the effect of Zn^{++} and Be^{++} ions on RNase digestion were studied. Zittle [37] has shown that zinc prevents the action of RNase on isolated nucleic acid *in vitro*. No decrease in the amount of material stained blue by May-Grünwald-Giemsa dyes resulted when fixed preparations of whole cells were incubated for 45 min. with RNase in the presence of 0.1 *M* zinc sulphate. A number of workers have described the precipitation of nucleic acids and nucleoproteins by beryllium ions [6, 15, 33] and Jacobson and Webb [17] found that Be^{++} ions readily penetrated into unfixed chick embryonic fibroblasts and rendered the nucleic acids more resistant to the action of nucleases. Washed cells of *Strigomonas oncopelti* were treated with beryllium chloride (2 per cent w/v) for 15 min., then washed in saline and smears were prepared for treatment with RNase. As reported by Jacobson and Webb [17] for mammalian cells, this treatment rendered the RNA more resistant to enzymic digestion. These results support the conclusion that the removal of material staining blue with May-Grünwald-Giemsa dyes by treatment with RNase was due to specific enzyme action and was not a non-specific extraction due to, for instance, an increased solubility of nucleoprotein in the enzyme solution.

Treatment with perchloric acid and sodium chloride solutions.—Extraction of fixed "smears" of organisms with *N* perchloric acid or with 0.14 *M* sodium chloride for 24 hr at 5°C caused a considerable loss of material staining blue with May-Grünwald-Giemsa dyes from both the cytoplasm and bipolar bodies; control preparations treated with glass distilled water under the same conditions lost little blue-staining material. Both *N* perchloric acid and 0.14 *M* sodium chloride are known to extract RNA under these conditions [25, 27].

Mitochondrial stains.—Janus Green B and Triphenyltetrazolium chloride







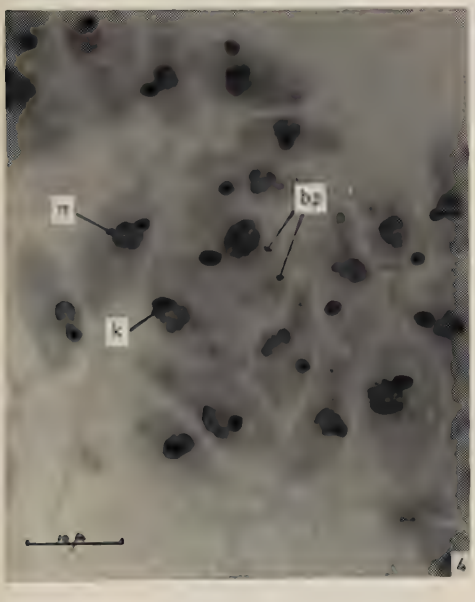


Fig. 1.—Phase contrast photomicrograph of unfixed cells of *Strigomonas oncopelti*. Organisms were suspended in 2.5 *M* sucrose and squashed between a cover glass and microscope slide by gentle pressure: bipolar bodies (*bp*), nucleus (*n*), kinetoplast (*k*).

Figs. 2 and 3.—Methanol fixed preparation, stained with May-Grunwald-Giemsa dyes: nuclei (*n*), red; kinetoplast (*k*), purple-red; bipolar bodies (*bp*), dark blue; cytoplasm, light blue.

Fig. 4.—Methanol fixed preparation, treated with ribonuclease for 15 min. at 37° C and then stained with May-Grunwald-Giemsa dyes. Ribonuclease treatment removed all blue staining material from cytoplasm and bipolar bodies, nuclei and kinetoplast were unaffected.

Fig. 5.—A purified preparation of bipolar bodies, methanol fixed and stained with May-Grunwald-Giemsa dyes.

(TTC) were used to locate the sites of oxido-reduction reactions in cells of *Strigomonas oncopelti*. Washed suspensions of organisms were suspended in 0.85 per cent sodium chloride buffered to a pH value of 7 by *M*/30 phosphates and containing 0.5 per cent glucose and either 0.01 per cent TTC or 0.001 per cent Janus Green B. Suspensions were incubated at 25°C and samples were removed for examination under the light microscope. After 1 hour in the presence of TTC small, faintly red granules were observed in the cytoplasm of the organisms, they were generally located at the periphery of cells and were not associated with the bipolar rods. No particles were stained by Janus Green B in living organisms.

Effect of growth conditions and starvation on the presence of bipolar bodies in Strigomonas.—Bipolar bodies have been observed in organisms in the lag, logarithmic and stationary phases of growth in peptone glucose medium and in a simple synthetic medium [23]. Incubation of washed suspensions of organisms in a buffered salts-glucose medium in the presence or absence of phosphate for periods of time up to 10 hours did not result in the loss of these structures from the cells. These results suggest that the bipolar bodies in *Strigomonas oncopelti* differ from the 'volution granules' which have been described in many protozoa and which have been considered as cytoplasmic reserve substances [32].

Observations on isolated bipolar bodies.—Bipolar bodies were isolated from other cell components by the techniques described above. Centrifugation of broken cell preparations through a medium of graded sucrose concentration resulted in an accumulation of bipolar bodies in the 9 *M* sucrose layer, careful removal of this layer gave preparations which contained little contaminating material. Fig. 5 shows a typical preparation stained with May-Grünwald-Giemsa dyes. Preparations were also examined in the electron microscope after fixation in either formalin or buffered osmium tetroxide (Fig. 6).

Nucleic acid content of bipolar bodies.—The ultraviolet absorption spectrum of a suspension of bipolar bodies in 2.5 *M* sucrose showed a sharp maximum at 259 mμ and there was a shoulder in the curve at 280 mμ indicating the presence of nucleic acid and protein. Table II summarizes the absorption characteristics and phosphorus content of nucleic acid extracted from a preparation of bipolar bodies by 0.5 *N* perchloric acid at 70°C; similar data for nucleic acid extracted from whole cells of *Strigomonas oncopelti* and for

Fig. 6.—Electron micrograph of an isolated bipolar body; formalin fixed.
Fig. 7.—Electron micrograph of isolated bipolar bodies after 30 min. fixation in buffered osmium tetroxide followed by 24-hour extraction with 0.14 *M* sodium chloride at 5°C.

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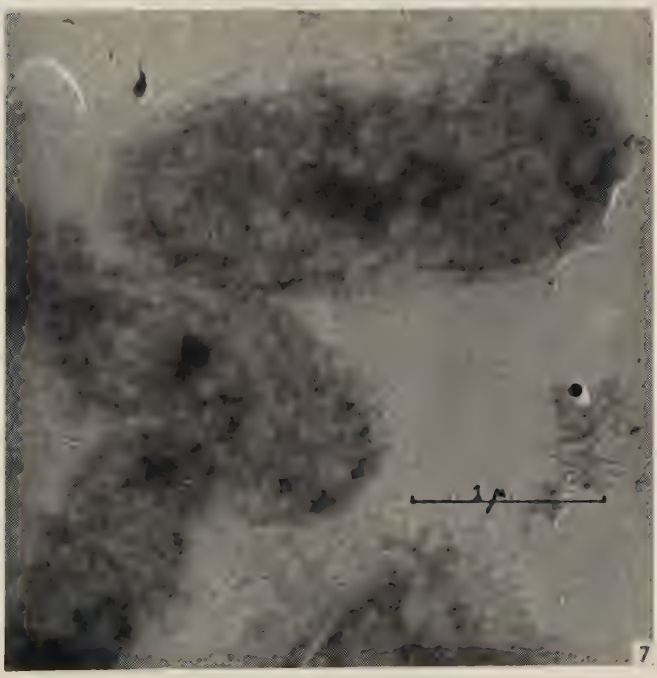
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TABLE II. *Absorption characteristics and phosphorus content of nucleic acids.*

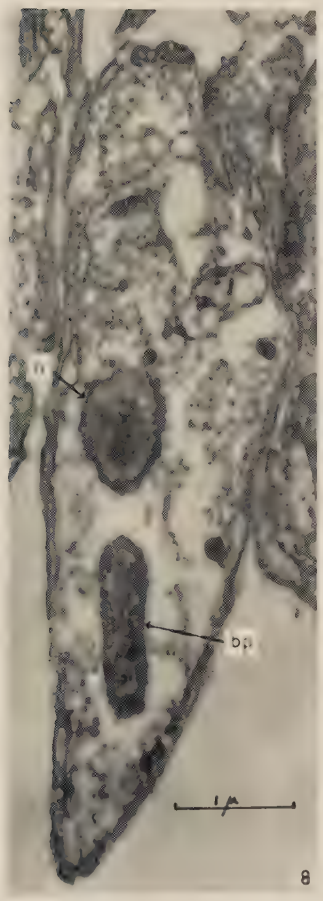
Source of nucleic acid	$E(P) \times 10^{-3}$ at 258 mμ	$E(P) \times 10^{-3}$ at 228 mμ	% phosphorus
Bipolar bodies	9.50	3.60	8.9
Whole cells	6.84	2.85	13.3
Whole cells*	9.3*	3.8*	9.1*
Yeast	9.8	3.5	8.6

* Values obtained after correction for excess acid-labile phosphate in the nucleic acid fraction.

The symbol $E(P)$ is used for the atomic extinction coefficient with respect to phosphorus as defined by Chargaff and Zamenhof [8], i.e. $E(P) = \frac{30.98\ E}{c\ 1}$ where E is the extinction (Log I/I) c is the concentration of phosphorus in g/litre and 1 is the thickness of the absorbing layer in cm.

a sample of yeast nucleic acid are also given in this table. The symbol $E(P)$ has been used for the atomic extinction with respect to phosphorus as defined by Chargaff and Zamenhof [10]. The values of $E(P)$ for the nucleic acid extracted from bipolar bodies and for yeast nucleic acid are in good agreement with the published figures for RNA from a number of sources [2, 9, 8, 12, 21, 29], indicating that nucleic acid is the sole important phosphorus-containing material in the perchloric acid extracts from bipolar bodies; the RNA content of bipolar bodies was estimated to be ca. 10–15 per cent of the RNA content of the whole cell. The values of $E(P)$ for nucleic acid extracted from whole organisms were considerably lower owing to the presence of excess phosphorus in this fraction. This excess phosphorus was found to be partly ortho-phosphate and partly acid labile phosphate which was converted to ortho-phosphate by hydrolysis with N HCl for 7 min. at 100°C. Since ortho-phosphate had already been extracted from the preparation by cold perchloric acid it seems probable that partial hydrolysis of the labile phosphate compound occurred during the extraction of nucleic acid by 0.5 N perchloric acid at 70°C. By deducting the ortho-phosphate + the 7 min hydrolysable phosphate of the nucleic acid fraction from the total phosphate of this fraction, as described by Wiame [36], the ratio $E(P)$ was found to be in good agreement with that for nucleic acid (Table II). No attempt was made to identify the acid labile compound, but it seems likely from the work of Wiame that it was metaphosphate; in support of this, cells of *Strigomonas oncopelti* were found to contain a number of small granules which stained

Figs. 8, 9 and 10. —Electron micrographs of ultra-thin sections of *Strigomonas oncopelti*. Sections show the variation in size of bipolar bodies (*bp*) and the development of a central constriction (*df*). Figs. 8 and 9 also show nuclei (*n*).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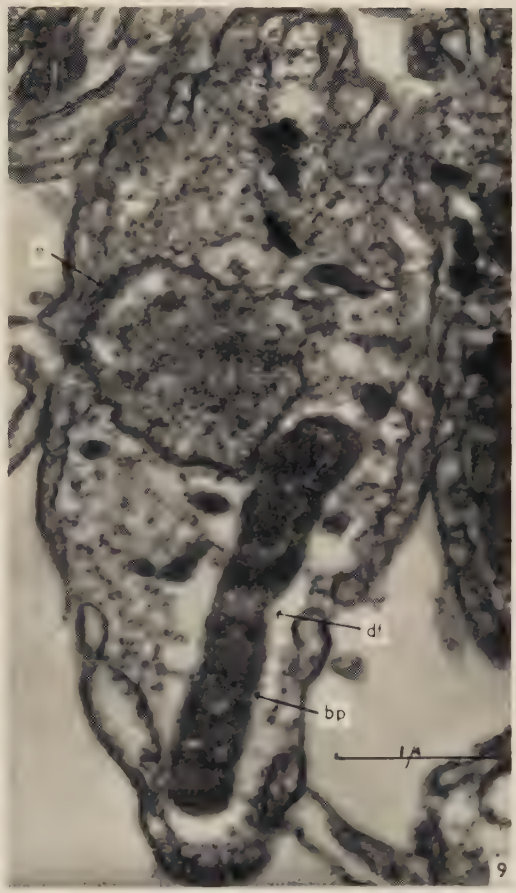
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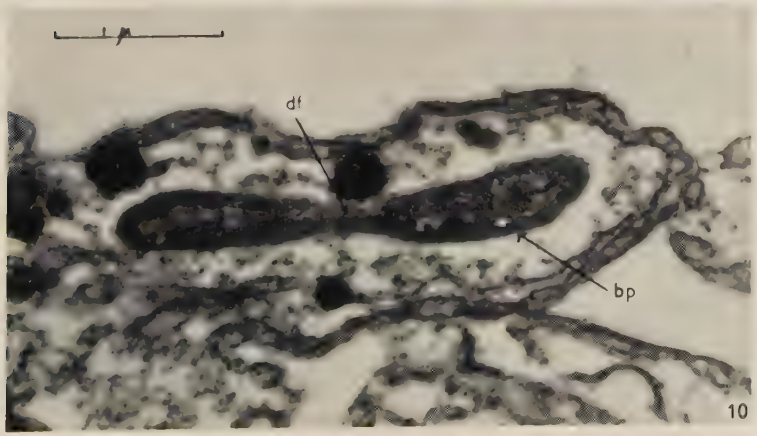
1 μ
df
bp
10

metachromatically with toluidine blue under the conditions described by Lindegren [20] for metaphosphate staining. The acid labile phosphate compound accounted for 16 per cent of the total phosphate content of cells and less than 0.4 per cent of their dry weight; none appeared to be associated with the bipolar bodies.

Cytochemical methods (Feulgen and methyl green stains) failed to show the presence of any deoxyribonucleic acid (DNA) in bipolar bodies when whole cells were stained. However, it was found that isolated particles were stained weakly by methyl green and the diphenylamine reaction showed the presence of small amounts of deoxyribose in the nucleic acid extracted from isolated particles. This DNA was probably due to contamination of preparations with small amounts of nuclear material. The weak methyl green staining of isolated bipolar bodies could be due to absorbed DNA from nuclei broken during the preparation but the possibility that these structures contain a small amount of DNA cannot be eliminated.

Protein content of bipolar bodies.—Determination of the total nitrogen content of bipolar bodies before and after extraction of nucleic acid has shown that they contain 15–20 per cent nucleic acid and 80–85 per cent protein; the possible presence of polysaccharide in the residues remaining after nucleic acid extraction was not taken into account in these calculations.

Evidence for a particle membrane.—Bipolar bodies were found to be sensitive to osmotic shock and to the surface active compound deoxycholate, which is known to disrupt the lipoprotein membranes of red blood cells and of mitochondria [30]. A direct demonstration of the presence of a membrane has been possible by electron microscopy. When isolated bipolar bodies were fixed in buffered osmium tetroxide for 30 min. and then extracted with 0.14 *M* sodium chloride for 24 hours at 5°C it was found that most of the electron dense material had been extracted from these structures without the destruction of the boundary membrane (Fig. 7).

Electron microscopy of ultra-thin sections of Strigomonas oncopelti.—Figs. 8, 9 and 10 show the appearance of bipolar bodies in ultra-thin sections of *Strigomonas oncopelti*. In Fig. 8 there is no sign of a 'division furrow' in the bipolar body whereas in Fig. 9 the bipolar body is nearly twice the length of the one in Fig. 8 and shows a slight central constriction and an area of low electron density; this section also shows a well defined membrane round the bipolar body. Fig. 10 shows what is probably a later stage in which there is a well marked central constriction. The variations in size and the presence or absence of a central constriction are also readily seen in the stained preparations of isolated particles (Fig. 5).

DISCUSSION

It seems likely, from the results described in the present paper, that the bipolar bodies which occur in the cytoplasm of *Strigomonas oncopelti* are self-reproducing structures. Cells have been observed to contain one or two, but never more than two, bipolar bodies. Studies of unfixed preparations by phase contrast microscopy, of stained preparations by light microscopy and of isolated particles by electron microscopy all suggest that division of these structures occurs by transverse fission; electron micrographs of ultra-thin sections, however, have failed to show the presence of an in-growing cross wall of the type seen in sections of dividing bacteria [3, 7]. It is not yet possible to say at what stage in the division cycle of the cell the bipolar body divides, or even if its division has any fixed relationship in time with nuclear division; many cells, however, have been seen which contain two bipolar bodies but show no sign of cytoplasmic, kinetoplast or nuclear division, (Figs. 2 and 3).

The results described in the present paper do not permit any conclusions to be drawn, or even any speculations to be made, about the physiological function of the bipolar bodies; in view of their high ribonucleoprotein content, however, it is thought that these structures might provide interesting material for the study of nucleic acid and protein synthesis at a sub-cellular level and work along these lines is now in progress.

SUMMARY

Basophilic, rod-like particles have been observed in the cytoplasm of the trypanosomid flagellate *Strigomonas oncopelti*. These structures have been observed in unfixed preparations by phase contrast microscopy; organisms contain either one or two of these structures which are always located at the posterior end of the cell. They are present in organisms harvested at all stages of growth, and starvation does not result in a loss of these structures. The results of cytochemical tests suggest that ribonucleic acid is associated with the particles; there is no evidence for the presence of polysaccharide or metaphosphate.

Particles have been liberated from cells and separated from other cell components. Analysis of isolated particles shows that they are composed largely of ribonucleoprotein and contain ca. 10 15 per cent of the total ribonucleic acid of whole organisms.

Electron microscope studies of isolated particles and of ultra-thin sections of whole organisms show that these rod-like structures are bounded by a thin membrane and that they probably divide by transverse fission.

The authors are indebted to Dr. W. Jacobson, of the Strangeways' Research Laboratories, Cambridge, for help and advice in preparing and photographing stained preparations; also to Mr. W. Grimstone, of the Zoology Department, Cambridge, for assistance in cutting some of the sections. The electron microscopy was carried out in the Electron Microscope section of the Cavendish Laboratory, Cambridge, under the supervision of Dr. V. E. Cosslett, with the Siemens 'Elmiskop' Electron microscope purchased through the generous benefaction of the Nuffield Foundation. One of us (R. W. H.) is indebted to the Agricultural Research Council for financial aid.

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SOME TRANSMISSION CHARACTERISTICS OF THE TUBULE-INDUCING INFLUENCE ON MOUSE METANEPHROGENIC MESENCHYME

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Metanephrogenic mesenchyme from the 11-day mouse embryo, separated by trypsin from the normally associated ureteric bud, can be induced by embryonic dorsal spinal cord to form epithelial tubules *in vitro* [2]. The inductive influence crosses thin membrane filters [4] in the absence of cytoplasmic contact [6]. The nature of the inductive influence has not been further specified, though the body of available data has encouraged speculation that it may involve macromolecular materials, probably including proteins [3, 5].

In the experiments which are reported here the distance between the interacting tissues has been increased by employing single thicker filters, or by layering several filters of lesser thickness. Further, cellophane with or without a perforation, has been incorporated as one of the layers between the interacting tissues. The results indicate that tubule-inducing activity, under the conditions specified, is limited in range to a distance of about 80 microns, that it cannot cross a cellophane membrane of 20 microns thickness, but that it can cross a fluid interspace of 20–35 microns thickness.

MATERIAL AND METHODS

General procedures have been described earlier [4]. The membrane filter discs[3] were rim-mounted on plexiglas rings and supported on two glass rods over a well in a flat tissue dish. The interacting tissues were superimposed on either side of the filter, each in its own plasma clot. With thicker filters of reduced translucency the tissues were oriented in relation to a reference mark on the rim in order to ensure accurate superimposition. The nutrient medium, changed daily, was horse serum,

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[3] Millipore Filter Corp., Watertown, Mass.

Tyrode's and chick embryo juice (2:2:1), added under the filter assembly as a "standing drop" between the assembly and the well. The cultures were incubated at 37°C in an atmosphere of 5 per cent CO_2 saturated with water vapor. Penicillin, streptomycin (5 mg each per 100 ml) and mycostatin (Mycostatin Squibb, 100 units per ml) were incorporated in the nutrient medium. The cultures were examined daily by transmitted and reflected light and uniformly fixed on the third day.

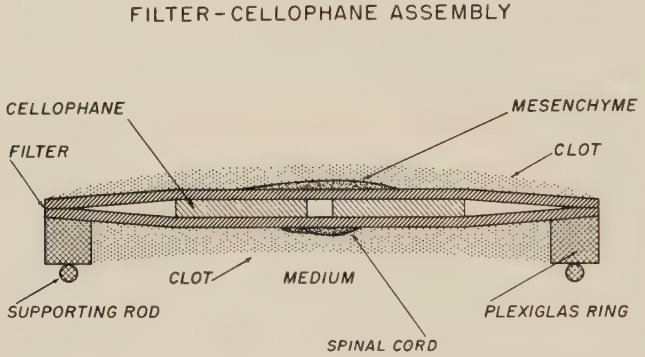


Fig. 1.—Diagram of filter-cellophane assembly.

To prepare multiple layers the filter discs were stacked and cemented together to the plastic ring. When cellophane was used, a disc, somewhat smaller than the inside diameter of the plastic ring, was included between two filter layers prior to cementing (Fig. 1). The discs were cut out of a cellophane sheet (Dupont, 300 PT or 600 PT) previously punched with holes of 0.1, 0.15 or 0.2 mm diameter. Each disc included one hole near its center and the tissues were superimposed so as to include the hole between them. The spinal cord fragment in most cases was larger than the hole (0.2–0.4 mm) and projected well beyond it, allowing simultaneous comparison of effects with and without intervening cellophane. Large-molecular materials presumably could exchange between the medium and the overlying layer through the filter layers peripheral to the entire cellophane disc.

RESULTS

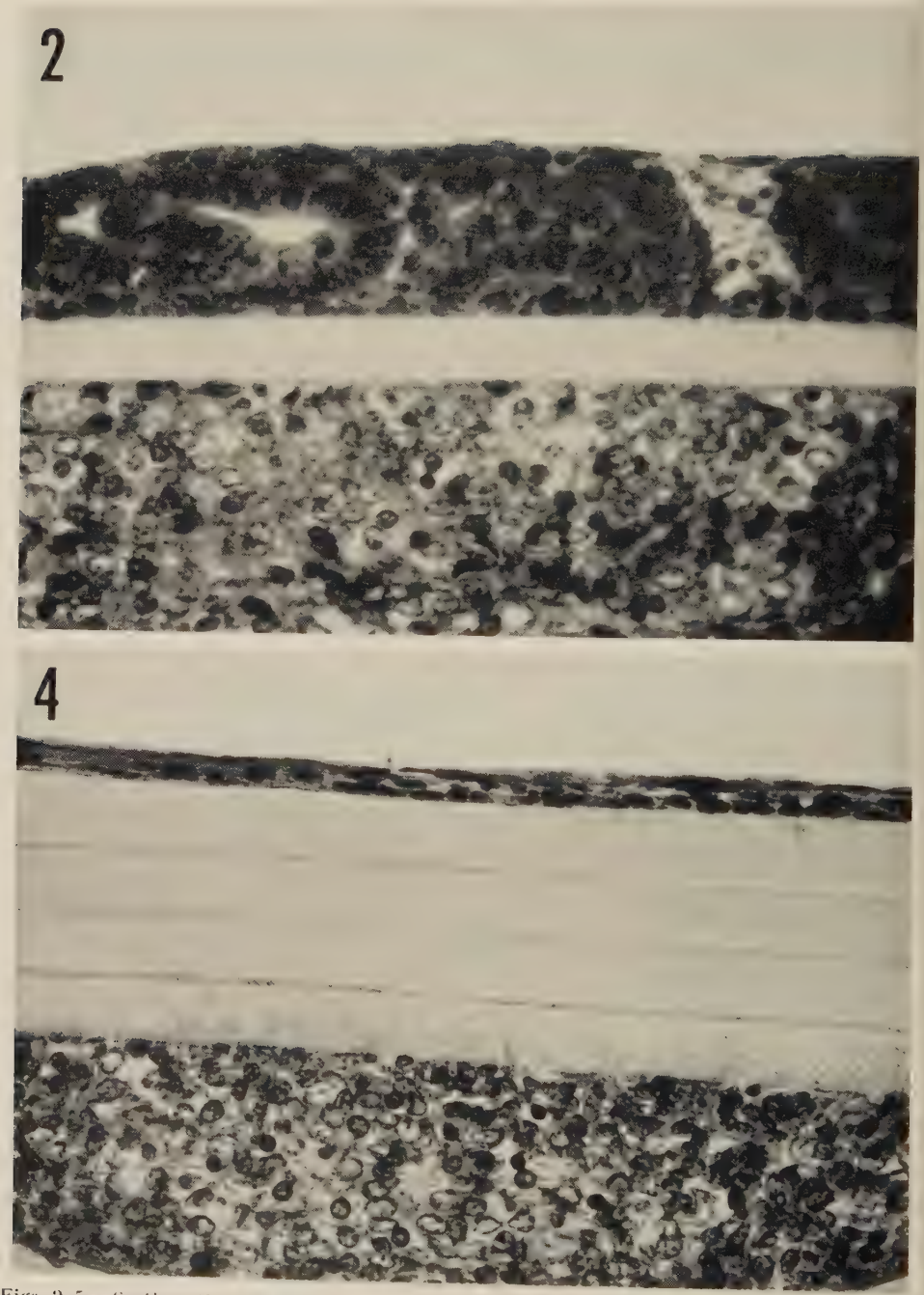
Transmisson distance.—Data on transmission distance were obtained from 6 experiments involving filters of varying numbers of layers or of varying thickness. Filters of either of two porosities were used (HA or AA), both previously shown to transmit inductive activity but differing in the amount of cytoplasmic penetration permitted [6]. Porosity determinations were not made on the particular samples -in most cases specially prepared thin filters—which may have varied in absolute values, though probably not in relative values, from the 0.45 ± 0.02 microns for HA and 0.8 ± 0.05 microns for AA specified by the manufacturer for commercial filters.

Filter samples were selected preliminarily as to thickness on the basis of direct measurements by the manufacturer on dry filters. The final thickness data given below, however, were obtained by measurement of filter cross-sections in cultures fixed on the third day of incubation, and serially sectioned and mounted *in toto*. The measurements, in relation to the actual distance separating the two tissues during their interaction, are subject to two possible sources of error: (1) deviation of the plane of section from a perpendicular to the filter surface, which would tend to increase the values; (2) shrinkage of the filter during processing, which would tend to decrease the values. The measurements in sections in all cases gave values 10–15 per cent lower than those expected from the preliminary estimates, which could be due to shrinkage. In any event, the indicated transmission distances cannot be regarded, in absolute terms, as more than an approximation of the actual.

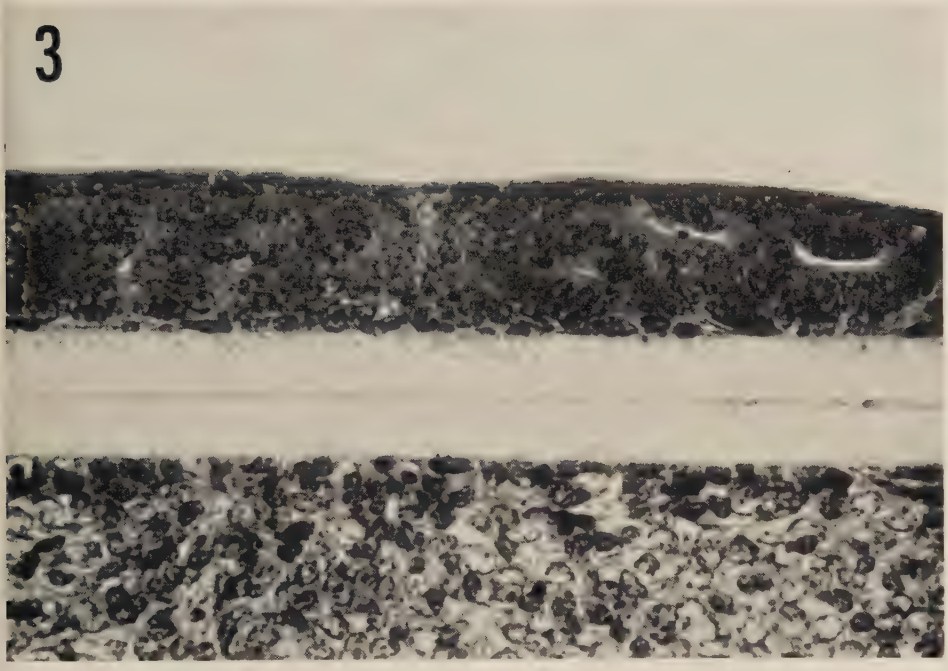
Induction effects were judged and measured by presence and number of epithelial tubules, by their level of differentiation, and by the maximum height or thickness of the responding tissue in sections. These three criteria showed close correlation, and mesenchymal thickness proved most convenient for quantitative treatment. It was determined for each case as the average maximum height for three sections: the median section of the series through the spinal cord, and the fifteenth section on either side of it (75 microns on either side).

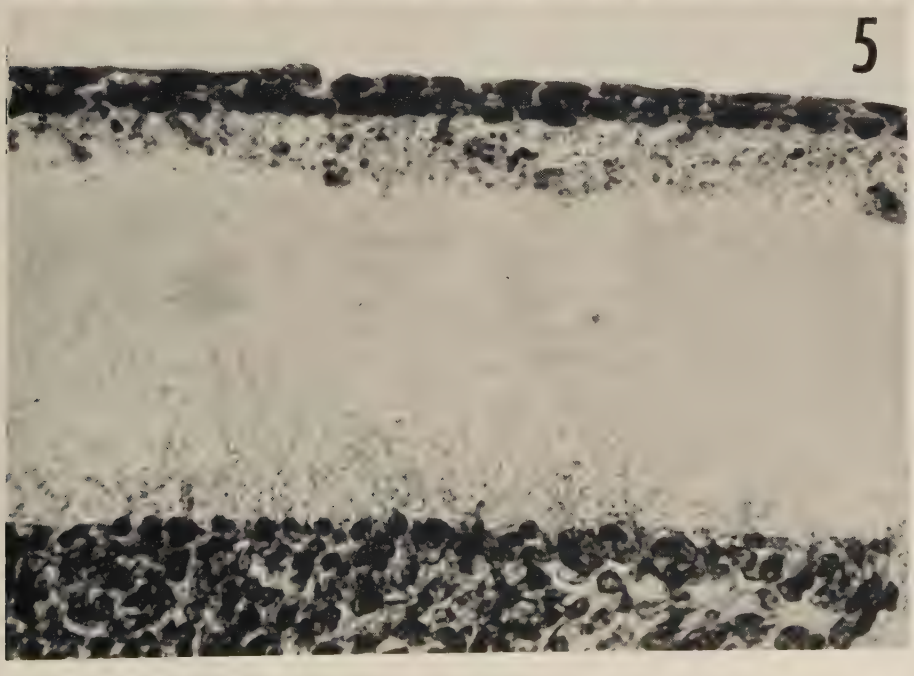
Thick commercial HA and AA filters were first compared with the thin (about 20 microns) TA-filters earlier found to give maximum transmission. Four cultures of each of the three filter types were prepared. With TA-filters the mesenchyme in three of the four cultures produced a number of well-formed tubule rudiments. In the fourth TA-culture the spinal cord was found not to be in contact with the filter and no induction occurred. In the eight cultures involving thick filters no tubule rudiments were produced after 3 days, as observed either in the living state or in fixed and stained sections (Fig. 5). The mesenchyme was spread as a thin sheet, one to three cell layers thick. Cytoplasmic penetration into the thick filters, particularly the AA-type, was considerably more abundant than with the thin filters, either in this experiment or in those previously described. Fine ramifying processes reached halfway or more through the filter from the spinal cord side. The mesenchymal penetration was shallower but coarser, and even included a few whole cells. Despite the greater cytoplasmic penetration, suggesting higher porosity of the thick filter, no inductive activity was evident.

Three experiments were then run in which the intervening filter zone consisted of 2–6 layers of thin TA or TH filters (Figs. 2–4). Of a total of 46



Figs. 2–5.—Sections through cultures fixed on third day of incubation, metanephrogenic mesenchyme above, spinal cord below. Figs. 2–4 – 1, 2, 4 TA-layers; Fig. 5 commercial AA-filter. Note thickness of mesenchyme and presence of epithelial tubules in 2 and 3, thinness of mesen-





chyme and absence of tubules in *4* and *5*, penetration of filter by cytoplasmic processes in all cases. ×330.

TABLE I. *Data on inductive transmission across AA-filters of different thicknesses.*

Exp. no.	No. cases	Filter thickness (microns)	Tubules		Mesenchyme thickness (μ)	
			Strong	Weak	Range	Average
B-175-1	5	19–20	5	–	23–41	30.4
-2	5	31–35	5	–	37–43	39.0
-3	5	47–52	–	5	13–27	21.6
-4	5	53–56	–	3	17–36	25.8
-5	5	60–65	2	2	22–29	24.8
B-217-1	4	35	4	–	33–42	35.0
-2	5	53–65	3	2	21–44	30.6
-3	3	76–82	–	–	12–17	14.8
-4	4	101–110	–	–	9–19	12.8

cultures, 17 included 1 or 2 layers and all were strongly positive. Of 10 cultures involving 3 filter layers, 7 were definitely positive, 2 were weak or questionable, and one was negative. Of 9 cultures of 4 filter layers, none was definitely positive, 4 were weak or questionable and 5 were negative. The weak responses in this group were all obtained with TH filters. Ten cultures involving 5 or 6 layers were negative.

Measurement of the filter layers in sections showed the TH-filter used in the experiments to be somewhat thinner (ca. 14 microns) than the TA-filter (ca. 17.5 microns), possibly accounting for the difference in behavior in 4-layer cultures of the two (total thickness: TH – ca. 56 microns; TA = ca. 70 microns).

Finally, two experiments were run involving AA-type single filters of various thicknesses ranging from 20 to 110 microns. Results of these two experiments, involving a total of 41 cultures, are given in Table I and summarized in Fig. 6. Although the data show considerable scatter, particularly at intermediate filter thicknesses, the trend is clear and may be summarized as follows. Well-developed tubules practically always formed at filter thicknesses below 70 microns, and never formed above this thickness. Mesenchymal thickness declined from a maximum around 40 microns at filter thicknesses up to 30 microns, to a minimum of about 10–12 microns at filter thicknesses above about 80 microns. From these data the effective transmission limit, as indicated by tubule formation, is in the vicinity of 80 microns, with gradual reduction of effect between about 30 and 80 microns.

and only occasional weak reactions beyond 80 microns as indicated by mesenchymal thickness.

When measurements were made on multi-layer filters in the experiments already described, the data obtained were in general conformity with these conclusions. Average data for one multi-layer experiment have been included

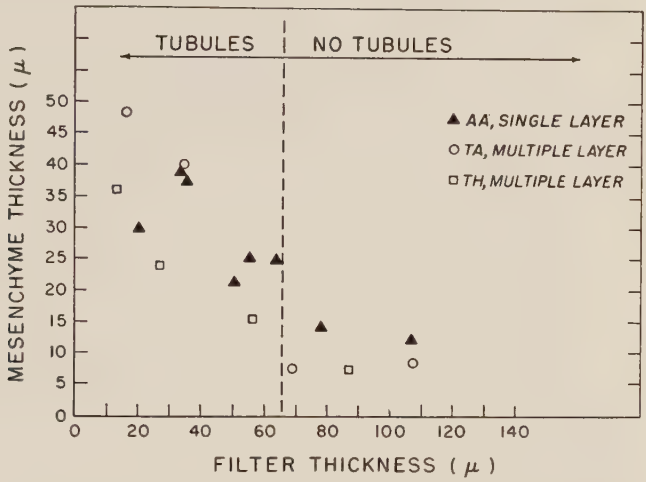


Fig. 6.—Mesenchyme thickness and tubule formation in relation to thickness of AA-type filters.

in Fig. 6. It will be noted that with multiple TA-filters, two (35 microns) and six layers (107 microns) behaved as expected in terms of their total thickness, but the four-layered (69 microns) cultures (only two cases) fell well below expectation. With multiple TH-filters a curve of the same general shape as with single AA-filters is suggested, but at a somewhat lower level throughout, possibly reflecting the lower porosity of the TH-filter. With the multiple filters, too, there were no cases of good tubule formation beyond 80 microns.

Delayed addition of mesenchyme.—It had been found previously (unpublished data) that the capacity of metanephrogenic mesenchyme to respond to a tubule-inductor declines with culturing, and is quite low by the third day. It seemed possible that failure to obtain induction with thick or multi-layered filters was due not to failure of the influence to pass across, but to decline of the response capacity of the mesenchyme before the influence arrived. This possibility was tested by delayed addition of mesenchyme in two experiments involving 8 single commercial filters (4 HA; 4 AA), and 12 cultures of 4-layered thin TH-filters. In the former case spinal cord was pre-cultured on the filter for 1 or 2 days, in the latter case for 0, 2, or 4 days, before addition of mesenchyme to the opposite side. All cases involving

delayed addition of mesenchyme were negative for induction, while three of the four cases in which mesenchyme had been added immediately to 4-layer cultures gave weak positive reactions. It does not seem likely that declining mesenchymal responsiveness is responsible for failure of induction through thick or multi-layered filters.

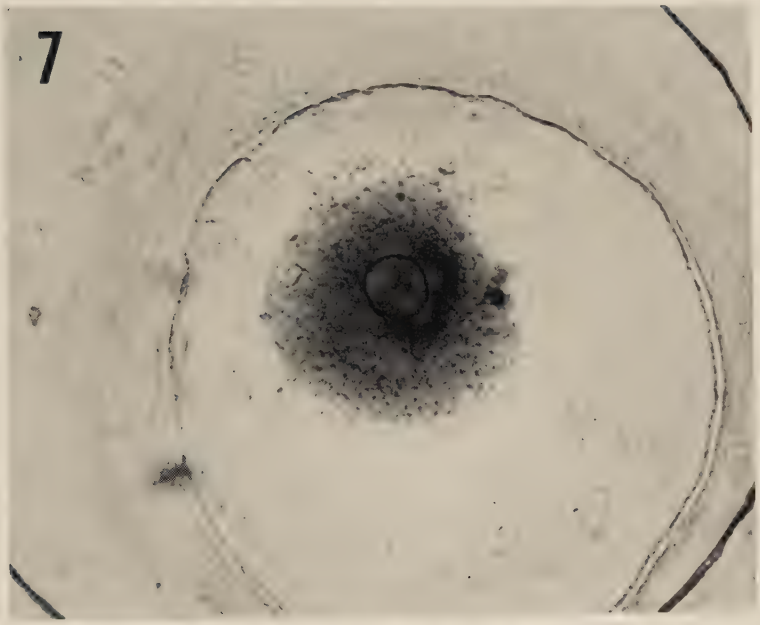
Cellophane barrier.—The fact that tubule-induction could be obtained across three thin filter layers made possible experiments with cellophane as an intermediate layer. Two objectives were in mind: first, to determine whether cellophane would block transmission as it has been reported to do in other inductive systems *in vivo*, and second, to make use of this property, if it existed, to provide a restricted volume through which the active material would be known to move, and in which it might be manipulated and further characterized.

In 4 experiments cellophane discs of one of three thicknesses, perforated by a hole of either 0.1 or 0.15 mm diameter, were interposed between the two TH filter layers. Four cultures involving cellophane of 125 microns thickness failed to show any sign of tubule induction or of residual thickness above the hole, i.e. the mesenchyme spread in a thin sheet as in control cultures without spinal cord below. However, when cellophane of 20 micron thickness perforated by a 0.15 mm hole was used in 9 cultures, definite inductions were obtained over the hole in five cases and weak reactions in two others.

A localized reaction over the hole was evident by 24 hours, when the peripheral mesenchyme was spreading and thinning while that in the vicinity of the hole remained thicker. By 48 hours the mesenchyme was a thin sheet everywhere except in the vicinity of the hole, where a small hillock or protrusion was centered on the hole but with its slopes extending a tenth of a millimeter or so beyond it. No tubule rudiments were recognised in the living cultures at this stage, but two cultures which were fixed and stained as whole mounts showed early rudiments in formation. By the third day, one to three tubule rudiments usually were observed over the hole or its periphery in the living cultures, and were definitely demonstrable in fixed and stained whole mounts (Figs. 7, 8). The presence of tubules was confirmed in sections of two cultures, but their localization could not be as certainly established because of difficulties in keeping the cellophane and filters in their normal relations

Fig. 7.—Tubule formation in relation to hole in cellophane layer between two HA-filters. Living culture, third day of incubation. Note three light areas over smallest circle marking edge of hole. Outermost dark circle is cellophane edge. ×55.

Fig. 8.—Culture in Fig. 7, fixed and stained whole mount. Edge of hole almost out of focus but visible under the three tubule rudiments. ×270.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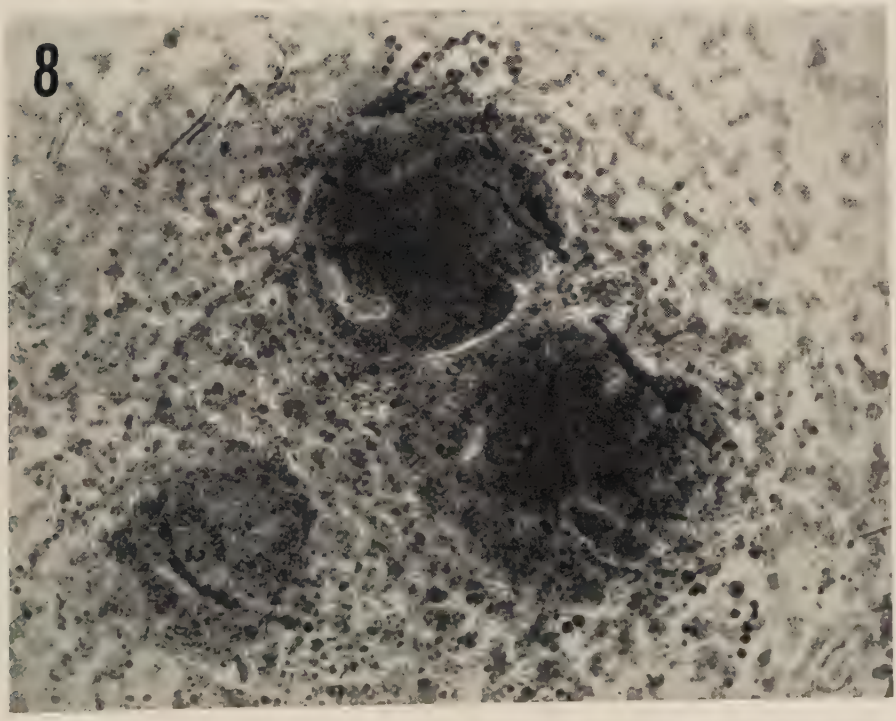
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TABLE II. *Relation of cellophane thickness and hole size to number of tubules induced.*

Cellophane thickness	Hole diameter (mm) 0.1	0.15	0.2	Total positive	Av. no. tubules
20 microns	1	0	0	6/12	3.3
	0	3	0		
	0	3	6		
	0	2	5		
35 microns	1	1	0	10/12	2.5
	1	4	5		
	1	2	3		
	0	1	6		
Total positive	4/8	7/8	5/8		
Av. no. tubules	1	2.3	5.0		

during the histo-technical procedures. Tubules never formed at any distance from the hole, i.e. over regions completely underlaid by cellophane, nor in control cultures involving unperforated cellophane.

In the four preliminary experiments, thicker cellophane (ca. 35 microns) or smaller holes (0.1 mm or less) gave negative or weak results. The latter usually involved local residual thickening centered over the hole, with small atypical condensations of mesenchyme or no evidence of tubule formation. When a fifth, definitive experiment was run, comparing cellophane thicknesses of approximately 20 and 35 microns and holes of 0.1, 0.15 and 0.2 mm diameter, results were obtained as shown in Table II. The difference between the two cellophane thicknesses does not seem significant, the thicker cellophane giving somewhat more positive cases but of slightly lower intensity as judged by average tubule number. On the other hand, size of hole clearly is significant, the number of positive cases being greater with holes greater than 0.1 mm, and the number of tubules formed in positive cases increasing with hole diameter.

It may be concluded that cellophane constitutes an effective barrier to the tubule-inducing material, but that the material can pass through the presumably fluid-filled space of up to 0.2 mm diameter and 35 microns height represented by the hole. The reaction in the overlying mesenchyme is limited to the area immediately over and around the hole, and as this area is in-

creased—within the limits tested—the number of tubules formed increases. It appears likely that some minimum amount of mesenchyme must be affected in order for a single tubule to form, and that this minimum is not achieved with holes much below 0.1 mm diameter.

DISCUSSION

The results strengthen the earlier conclusion, based on electron microscopic observations [6], that the inductive effect of dorsal spinal cord on metanephrogenic mesenchyme is not dependent upon cytoplasmic contact between the two tissues. Cytoplasmic penetration of low-porosity TV-filters was found to be virtually absent, yet inductive effects were obtained in reduced intensity across such filters of slightly less than 20 microns thickness. TH-filters, across which strong inductive effects take place, showed sparse cytoplasmic penetration largely limited to the first 6–8 microns of the filter thickness but infrequently occurring to greater depths. It was not felt safe to conclude that all possibility of occasional cytoplasmic contact in TH-filters of approximately 20 microns thickness was eliminated. However, induction now is found to take place regularly across 3 layers of TH-filters, with total thickness of almost 60 microns. Significant cytoplasmic penetration of this distance is contraindicated by the earlier electron microscopic observations. It thus seems certain that cytoplasmic contact is not involved in this particular induction through either TH or TV-filters. The same conclusion cannot be safely drawn from the behavior of TA-filters, where cytoplasmic penetration is abundant and may reach distances up to 50–60 microns. It appears unlikely, however, that the cytoplasmic penetration is any more essential to the induction with this filter than with the other two.

Maximum transmission distance, though possibly slightly less with TH than with TA filters, is of the same order of magnitude with both, whether in single or multiple layers. Keeping in mind the possible errors involved in the measurements, the data are in accord in indicating a maximum distance in the vicinity of 80 microns. It is to be noted that this is for filters in the porosity range of the TH and TA filters (0.4–0.8 microns?), and that the maximum range for TV-filters (0.1 microns?) appears to be considerably less, in the vicinity of 20–30 microns [6]. Further data are needed to confirm the suggestion that porosities between 0.4 and 0.1 microns may be significantly and progressively more limiting to passage of the material involved.

Meanwhile, the possibility that porosities in the vicinity of 0.8 microns are not limiting, and that "true" distance maxima which are physiologically

significant are being observed, is supported by the striking agreement of the *in vitro* data with those obtained *in vivo* in amphibia by Cambar [1]. Cambar deviated the Woffian duct so that it angled away from the mesonephric blastema, and later measured in sections the distance between the two at various antero-posterior levels, correlating these distances with the growth and differentiation of the mesonephric blastema at each level. At distances greater than 100–120 microns the mesonephros was undifferentiated. Some proliferative activity and weak differentiation was observed at 90–100 microns, but induction was not normal until the distance was approximately 70–80 microns. Allowing for the very unlike circumstances under which the estimates were obtained, the apparent agreement in two related but different inductive systems in mouse and amphibia is remarkable.

Efforts at physico-chemical interpretation of the data appear premature at the moment. Failure of the activity to pass cellophane is in accord with earlier impressions of involvement of a large molecular material, whether as a direct mediator or as a carrier. The fall of activity with distance can be regarded as a simple concentration gradient, but the usually strict localization of inductive effects within the limited distances available in inductive systems, together with the complexities suggested by recent studies [7, 8, 9] on the integrative mechanisms of slime molds, warn against taking too seriously so simple an assumption. Data on stability of the active material, production rate, rate of movement, and response time of the mesenchyme are needed before profitable hypotheses can be formulated as to the details of mechanism. The collection of such data may be facilitated by the cellophane layer procedure, since the induction can be interrupted or started at will by shifting the cellophane, and the possibility exists that material in the hole may be able to be shifted away from the source for detection and measurement of activity.

SUMMARY

1. When the distance between embryonic dorsal spinal cord and mouse metanephrogenic mesenchyme, undergoing inductive interaction *in vitro*, is increased—either by varying the thickness of single filters or layering several filters between them—induction decreases in intensity between about 30 and 60 microns and is largely eliminated at about 80 microns.

2. The distance limitation persists even when addition of the mesenchyme is postponed for 1–4 days, indicating that the activity is not simply delayed by greater distance but actually does not traverse it.

3. The active material does not cross cellophane, but does move through a fluid-filled hole in the cellophane producing inductive effects immediately over and surrounding the hole. The procedure is suggested as possibly advantageous for obtaining additional information on the nature and mode of action of the inductive material.

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BRIEF NOTES

THE ELECTRON DISTRIBUTION IN SOME HIGH ENERGY PHOSPHATES AND THE TRANSFER OF ENERGY FROM CATABOLISM TO ANABOLISM

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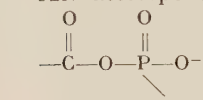
An attempt to calculate the distribution of six dislocalized π-electrons in the



group of high energy phosphates has been carried out. The calculation shows that the π-electron density of the central O atom is diminished, rendering this atom positively charged. However, since O atoms tend to attract electrons, it is probable that the electrons in the σ-bonds from the central O atom towards the C atom and the P atom become attracted closer to the O atom than in the free carboxyl and phosphate ions. Such an attraction will result in a weakening of the σ-bonds. Tentatively the process can be described as follows: One force tends to result in the formation of the electron distribution of the carboxyl ion, which represents a low energy state. This implies, however, that electrons must be drawn from the P O bond, thus giving an activated $^{+}PO_3$ group. But at the same time another force tends to produce the electron distribution of the phosphate ion, which in the same manner causes an activation of the keto group. Since, in such compounds, either the C–O bond or the P–O bond can be split, according to experiments by e.g. Boyer *et al.* [1], Cohn [2] and Harrison *et al.* [3], it can be suggested that the two above-mentioned forces will balance each other in the free molecule. When a specific perturbation is effected, e.g. in the form of a specific enzyme, one of the forces will predominate. Let us assume that the electron distribution is displaced towards the carboxyl ion so that the P–O bond is weakened. In such a case, the phosphoryl group will be activated and able to react with ADP,[1] the splitting of the weakened P–O bond being thus accomplished. This is in accordance with experimental results (e.g. [1, 2, 3, 4]) that the group, to which the O atom goes after the rupture, drops to a lower energy level.

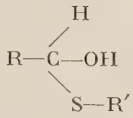
There are three characteristics of the high energy phosphates with the group in question, which may render them suited for transfer of energy from catabolism to anabolism:

[1] The following abbreviations are used: ADP for adenosine diphosphate, ATP for adenosine triphosphate, and DPN^{+} for diphosphopyridine nucleotide.

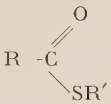
(1) The coupling between the dislocalized electrons and the σ-electrons, which means that the decrease in energy of one part of the molecule causes the activation of the other part. (2) The fact that, depending on the mode of perturbation, either the C–O or the P–O bond can be broken. (3) The balance between the above-mentioned forces, which means that only a specific perturbation can activate the group. This is a device which may preserve energy for anabolic purposes.

These characteristics depend on the presence of the two double bonds on each side of the central O atom with its lone pair of electrons. Properties (1) and (2) are prerequisites for transfer of energy from one group or substance to another.

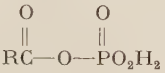
In certain instances of cellular energy transfer, aldehydes are connected to thiol groups prior to oxidation. It is probable that the thiol complex is added to the aldehyde, so that



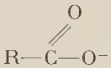
is formed [7]. By oxidation a H^- is removed from the aldehyde–thiol complex, by e.g. DPN^+, and this causes that the hydrogen bound to the oxygen is removed as H^+ [8], giving

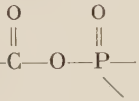


By reaction with a phosphate ion the $RC^+{=}O$ group and the phosphate ion give

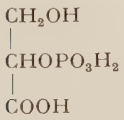


When this substance is perturbated, resulting in a splitting of the P–O bond, the keto group receives two electrons and an O atom, thus forming a carboxyl ion,

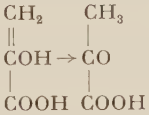


In this last reaction step, the redox state of the keto group is unchanged but the energy is decreased. Thus the decrease of energy is not connected with the oxidizing step, viz. the removal of one H atom and an electron. Instead the energy decrease results from the last step, in which an oxygen atom and two electrons are added to the keto group. The first step is nevertheless necessary because it renders the keto group suited for a reaction with a phosphate ion with formation of the  group. As is seen above a group of such a character is necessary for the transfer of energy from one group to another.

It can be of interest to compare the above process with the energy transfer from 2-phospho-glyceric acid,

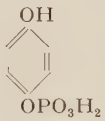


to ATP. In this case it is the energy difference [6] between the enolform and the ketoform of the pyruvate,



which will be conserved as chemical energy in ATP. It is the removal of H_2O from the 2-phospho-glyceric acid which gives a compound with such properties that a decrease in energy of the pyruvate group causes an activation of the phosphoryl group. It can be suggested that a perturbation of the pyruvic-enol-phosphate will change the electron distribution towards the ketoform. This may cause a weakening of the P–O bond and thus an activation of the phosphoryl group.

There is some reason to presume that hydroquinones may be a link in the energy transfer in the cytochrome part of the respiration chain [5]. Therefore it can be of interest to make a tentative explanation of this process. Let us assume that a compound



can be formed. This formation of a C-O–P link requires energy, which may be gained by the oxidation of the preceding carrier. It is probable that a removal of two π-electrons from the benzene ring can cause an attraction of the σ-electrons of the O-H bond and the O-P bond in the *para* substituent, thus activating the phosphoryl group. If e.g. ADP is present it is probable that the reaction can continue and that ATP, an H^+ ion and a quinone can be formed. In this case the oxidation is brought about by removing two electrons from the benzene ring, this step being directly connected with the splitting of the P–O bond and the change in energy.

The above discussion concerning aldehydes and hydroquinones may show that it is an intimate relation between the kind of oxidizing substance and the kind of oxidation that is necessary for the production of the active phosphate complex. In the former case, DPN^+ removes an H atom and an electron from the aldehyde complex. In the latter case, it can tentatively be presumed that the Fe ions of the cytochrome complex remove two electrons from the hydroquinone phosphate.

A more detailed report will be published later.

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ÜBER DEN FEINBAU DER MITOCHONDRIEN DES SCHLEIMPILZES *BADHAMIA UTRICULARIS*

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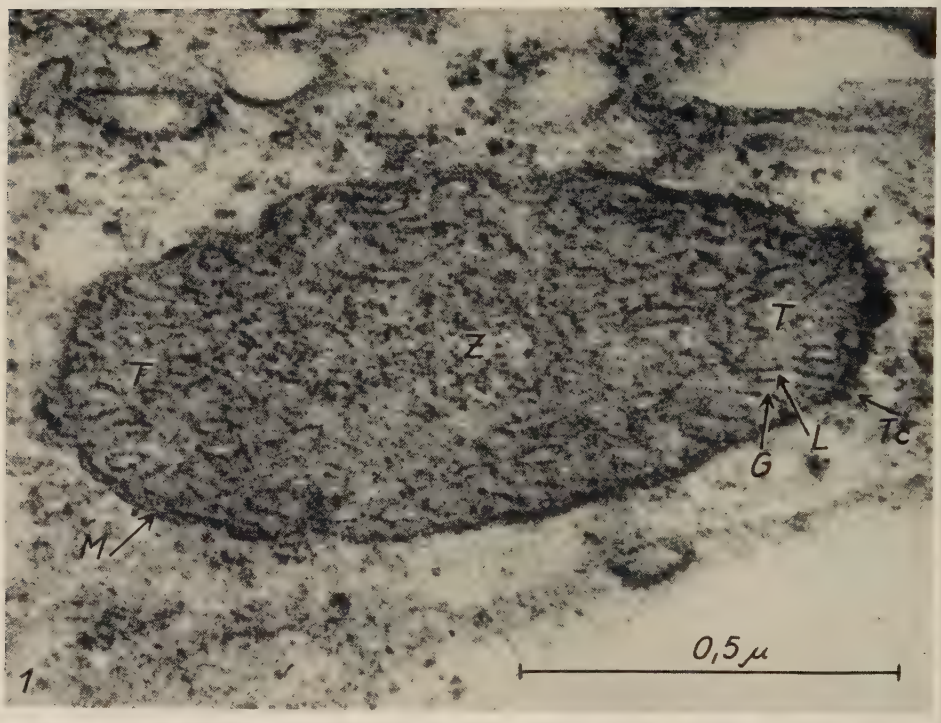
Durch die raschen Fortschritte in der elektronenmikroskopischen Präparationstechnik sind in den letzten Jahren auch die Mikroorganismen wieder häufiger Gegenstand cytomorphologischer Untersuchungen geworden. Elektronenmikroskopische Untersuchungen zur Feinstruktur der Myxomyceten liegen bisher nur an Totalpräparaten über die granulären Zelleinschlüsse von *Physarium polycephalum* vor [23, 24]. Ferner wurde über die chemische Zusammensetzung und den Feinbau der Membranen von *Dictyostelium discoideum* anhand ultradünner Schnitte und röntgenmikroskopischer Untersuchungen berichtet [5, 8]. Vorliegende Untersuchungen hatten die Aufgabe, die Feinbauverhältnisse der Mitochondrien dieser Organismengruppe näher zu analysieren.

Material und Methoden.—Zu unseren elektronenmikroskopischen Studien verwandten wir zunächst die Plasmodien von *Badhamia utricularis*[1]. Die Objekte wurden in einer 1%igen, gepufferten [14] OsO_4-Lösung (pH 7,4) für zwei Stunden fixiert und drei bis vier Stunden mit Formol (neutralisiert, 1:10 verdünnt) nachfixiert [10]. Die Polymerisation der Metacrylsäureester erfolgte im U.V.-Licht [12]. Die Ultradünnschnitte wurden mit einem Ultramikrotom eigener Konstruktion [9] hergestellt[2].

Ergebnisse.—Die Mitochondrien der Plasmodien von *Badhamia utricularis* besitzen eine eiförmige Gestalt und sie erscheinen demzufolge im Längsschnitt als ovale und im Querschnitt als runde Zellgebilde. Der mittlere Durchmesser der längeren Achse

[1] Für die freundliche Überlassung von Myxomycetenstämmen danke ich Herrn Prof. A. L. Cohen, Atlanta, Georgia, U.S.A.

[2] Für die Herstellung der Ultradünnschnitte danke ich Fräulein Waltraut Hilbert.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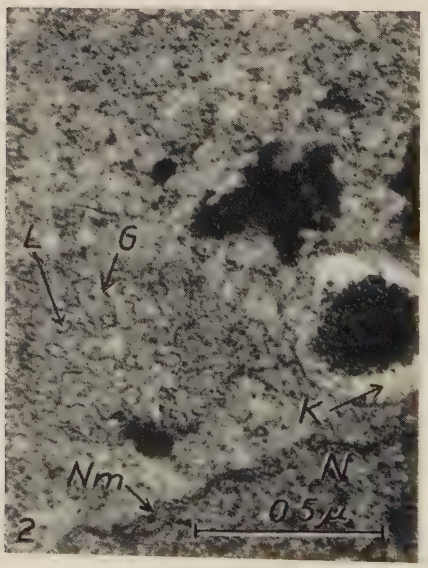
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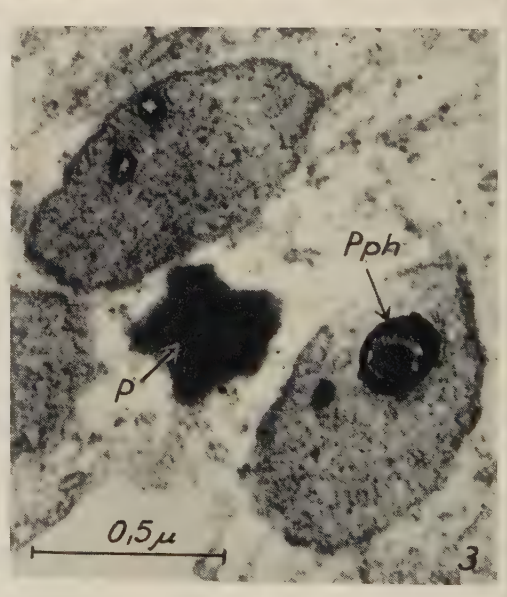
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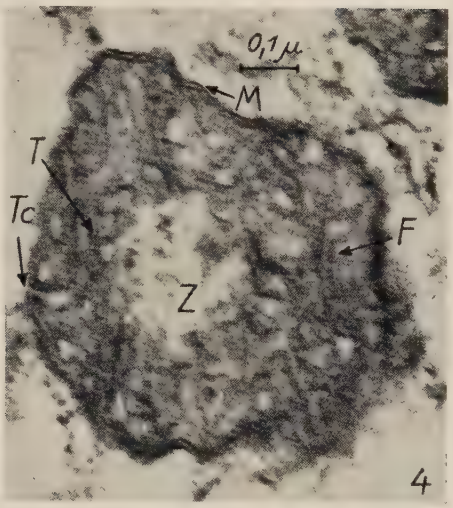

Abb. 4. — *Badhamia utricularis.* Querschnitt eines Mitochondriums. *F*, doppelt-konturierte Tubuluswand. Originalaufnahme 8700×, nachvergrößert.

beträgt 1 μ. Die Mitochondrien werden von einer doppelt konturierten Membran umgeben, die ungefähr 100 Å dick ist (Abb. 1, 4*M*). Während der Bereich des Zentrums eine feingranuläre Strukturierung erkennen läßt (Abb. 1, 4*Z*), so wird der übrige Mitochondrienraum von einer Vielzahl von schlauchartigen Gebilden ausgefüllt (Abb. 1, 4*T*). Beide beschriebenen Regionen gehen kontinuierlich ineinander über, ohne daß es hier zur Ausbildung einer besonderen Membran oder Grenzschicht kommt.

Der submikroskopische Feinbau der schlauchartigen Gebilde ist folgender. Im Längsschnitt begrenzen zwei stark osmiophile Grenzschichten (*G*) eine mittlere homogene und weniger konstrastreiche Zwischenlage (*L*), die man an Tangentialschnitten der Mitochondrien (Abb. 2) als das Lumen der intermitochondrialen Schläuche identifizieren kann. Ferner kann man besonders an solchen Tangentialschnitten eine ringförmige Anordnung der osmiophilen Grenzschichten beobachten (Abb. 2*G*). Sie stellen die Querschnitte durch die oben genannten schlauchartigen Gebilde dar.

Die einzelnen osmiophilen Grenzschichten wiederum besitzen eine charakteristische Feinstruktur. Auch hier wird eine kontrastarme Zwischenschicht von zwei kontrastreicheren Begrenzungslamellen umgeben (Abb. 4*F*). Während die osmiophile Grenzschicht insgesamt 80 Å dick ist, so hat das Lumen der Schläuche einen mittleren Durchmesser von 150 Å. In einigen Fällen konnten Verzweigungen der Schläuche

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Abb. 1. — *Badhamia utricularis.* Längsschnitt eines Mitochondriums. *M*, Membran; *Z*, zentraler Bereich; *T*, Region der Tubuli mitochondriales; *G*, osmiophile Grenzschicht der Tubuli; *L*, Lumen der Tubuli. Originalaufnahme 8700×, nachvergrößert.

Abb. 2. — *Badhamia utricularis.* Tangentialschnitt eines Mitochondriums. *K*, Nahrungsvakuole; *N*, Kern; *Nm*, Kernmembran. Originalaufnahme 8700×, nachvergrößert.

Abb. 3. — *Badhamia utricularis.* Mitochondrien mit Phosphatgranula verschiedener Größe. *P*, Pigmentgranula; *Pph*, Phosphatgranula. Originalaufnahme 8700×, nachvergrößert.

beobachtet werden. Auf Grund des oben beschriebenen Erscheinungsbildes können wir diese Strukturen der peripheren Bereiche der Mitochondrien als schlauchartige Gebilde ansprechen.

Diese Schläuche haben außerdem eine bevorzugte Richtung der Anordnung in den Mitochondrien. Sie verlaufen, mehr oder weniger gefaltet und verschlungen, schließlich radial zur begrenzenden Mitochondrienmembran hin. An geeigneten Schnitten kann man ferner erkennen, daß die Schläuche die begrenzende Mitochondrienmembran durchsetzen können (Abb. 1,4*Tc*), wobei gleichzeitig das Lumen der Schläuche zum Cytoplasma eine offene Verbindung besitzt; genauso, wie eine offene Verbindung zum homogenen Zentrum besteht.

Andererseits kann man in den Mitochondrien stark osmiophile Granula verschiedener Größe (von 50 mμ bis 500 mμ) beobachten (Abb. 3). Bei starkem Elektronenbeschuß zeigen sie Sublimationserscheinungen und weisen schließlich eine ring- oder netzförmige Strukturierung auf (Abb. 3), die dann trotz weiterem Elektronenbeschuß unveränderlich bleibt.

Diskussion.—Bisher wurde in der Hauptsache eine lamellige Feinstruktur (Cristae mitochondriales) der Mitochondrien tierischer Organismen beschrieben ([15, 19, 22] u.a.) (Die Beobachtungen an pflanzlichen Mitochondrien sind bis heute noch sehr lückenhaft, vergl. [5]. Daselbst Literaturzusammenfassung der bisherigen Beobachtungen an pflanzlichen Mitochondrien.) In der letzten Zeit konnte jedoch gezeigt werden, daß neben dem Bauprinzip der Cristae mitochondriales noch andere Bauprinzipien auftreten können [1, 3, 4, 16, 17, 18, 20, 21, 25, 26]. Es wurde eine „tubular structure" für Insektenmitochondrien beschrieben [16], wie sie auch bei Ciliaten gefunden [17, 18, 20, 21, 25, 26] und als „Tubuli mitochondriales" beschrieben wurde [25, 26]. Während einige Autoren keine begrenzende Außenmembran, dagegen aber eine offene Verbindung des Lumen der Tubuli mitochondriales mit dem umgebenden Cytoplasma beobachtet haben [17, 18], so beschreiben andere Autoren das Vorhandensein einer Außenmembran [20, 21, 25, 26]. Die Tubuli stellen dabei Einfaltungen der „inneren Membran" dar [20, 21].

Nach unseren Untersuchungen an den Mitochondrien des Schleimpilzes *Badhamia utricularis* können wir feststellen, daß bei diesem Objekt einerseits die Mitochondrien eine doppelt-konturierte Membran aufweisen und daß andererseits die Tubuli mitochondriales wahrscheinlich eine offene Verbindung zum Cytoplasma hin besitzen, wie dies schon für die Mitochondrien von Ciliaten beschrieben wurde [17, 18]. Die Tatsache, daß die Tubuluswand (osmiophile Grenzschicht der Tubuli) ebenfalls doppelt-konturiert ist, deutet darauf hin, daß die gesamte Außenmembran mit am Aufbau der Wände der Tubuli beteiligt ist, indem sie ins Innere der Mitochondrien Fortsätze bildet, die schließlich die Tubuli mitochondriales darstellen.

Wohlfarth-Bottermann schreibt ferner, daß diese Tubuli mitochondriales als geformte Sekrete ins Cytoplasma abgegeben werden, da man diese Strukturen u.a. dort ebenfalls beobachten kann. Unsere Beobachtungen jedoch, daß die Lumen der Tubuli mit dem Cytoplasma ein Kontinuum bilden und wir außerdem im Zentrum der Mitochondrien einen völlig anders strukturierten Bereich feststellen können, lassen vielmehr die Vermutung offen, daß im zentralen Bereich der Mitochondrien spezifische Stoffe gebildet werden, die dann durch das Lumen der Tubuli mitochondriales ins Cytoplasma gelangen. Dabei muß man aber auch einen entgegengesetzten Vorgang mit in Erwägung ziehen.

An Totalpräparaten von Myxomycetenplasmodien konnten Zellpartikel beobachtet werden [23, 24], die in ihrem Inneren stark elektronenstreuende Granula aufweisen. Nach unseren vorliegenden Untersuchungen können auf Grund ihrer Größe und Form jene beschriebenen Zellpartikel durchaus den Mitochondrien entsprechen. Die stark elektronenstreuenden Granula ([23] Abb. 1, 3*A*, *C*, *D*, *F*; [24] Abb. 2*A*, *B*, 3, 4, 5), die isoliert dieselbe Strukturierung aufweisen, wie wir sie ebenfalls für die stark osmiophilen Granula in den Mitochondrien beschrieben haben, können ebenfalls identisch sein. Die an anderen Organismen gemachten Beobachtungen [2, 3, 4, 7, 11] stützen unsere Vermutung, daß es sich bei diesen Granula um Zellgebilde handelt, an deren Aufbau kondensierte Phosphate (Metaphosphate? — Polyphosphate?) beteiligt sind, wie sie neuerdings bei zahlreichen Mikroorganismen nachgewiesen werden konnten. [13]. Diese Beobachtung ist insofern von enormer Bedeutung, als wir hier wahrscheinlich ein Objekt gefunden haben, das vielleicht nähere Aufschlüsse über die Identität von Mitochondrien und kondensierten Phosphatgranula und ihre Genese, einem aktuellen Problem gerade in der Mikrobiologie, geben kann.

Eine ausführliche Beschreibung unserer Ergebnisse über die Feinstruktur der Plasmodien von Myxomyceten erfolgt demnächst an anderer Stelle.

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A POLARIZING AND ELECTRON MICROSCOPE STUDY OF PLASMA CELLS[1]

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In electron micrographs of osmium tetroxide fixed plasma cells a relatively large amount of endoplasmic reticulum is regularly seen. This structure tends to be arranged in concentric layers beneath the cell membrane and would be expected, therefore, to display form birefringence. Here we present presumptive evidence, based on a comparison of polarizing and electron microscope observations, for the existence of this structure in the living cell.

Samples of human marrow were obtained by sternal biopsy from patients with multiple myeloma. From these, specimens were prepared for the electron microscope by fixation for two hours in 0.25 per cent buffered osmium tetroxide [5, 7] with potassium chloride added [6], by subsequent embedding in 9:1 normal butyl:methyl methacrylate, and by thin sectioning.

Fresh marrow preparations were made by compressing a small portion of marrow between slide and coverslip, and ringing the cover with vaseline. These were examined with a polarizing microscope designed and built by Dr. S. Inoué [4].

An electron micrograph of a typical plasma cell is seen in Fig. 1. The numerous cytoplasmic membranes, previously described by several authors [2, 3, 6, 9, 10], tend to occupy a peripheral zone of the endoplasm. The membranes appear to be arranged in paired, circumferential lamellae. Pairing of the membranes, their thickness, and the associated small particles serve to identify them as endoplasmic reticulum [8]. The relative abundance of this structure in plasma cells, the rapid rate at which plasma cells are presumed to synthesize protein, the strong basophilia and ultraviolet absorption in the cytoplasm of these cells [1] support the hypothesis that nucleic acid is concentrated in the endoplasmic reticulum and that this structure is a site of protein synthesis. With the polarizing microscope (Figs. 2 and 3) this structure is not resolved. Mitochondria tend to occupy a more central region (Figs. 1–3) which is probably analogous with the halo seen in stained preparations. Occasional clusters of small vesicles (Fig. 1) suggest the presence of a Golgi network.

This peripheral zone exhibits a weak birefringence with retardations between 20 Å and a minimum detectable value of 0.3 Å. The sign of the birefringence is positive with respect to a radial axis.[2] The birefringent areas in Figs. 2 and 3 may be identified by noting regions in which a 90° rotation of the specimen results in a reversal of

[1] This work was aided by a grant from the United States Public Health Service.

[2] Dr. Inoué, now at the University of Rochester, has kindly confirmed these observations on myeloma marrow, and has also found the sign of the birefringence to be radially positive.

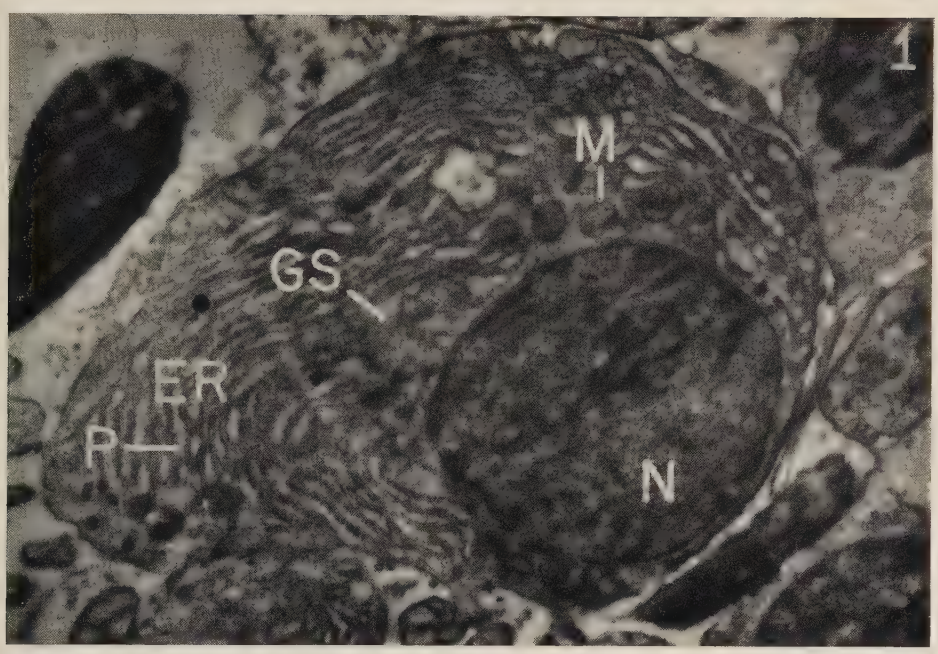
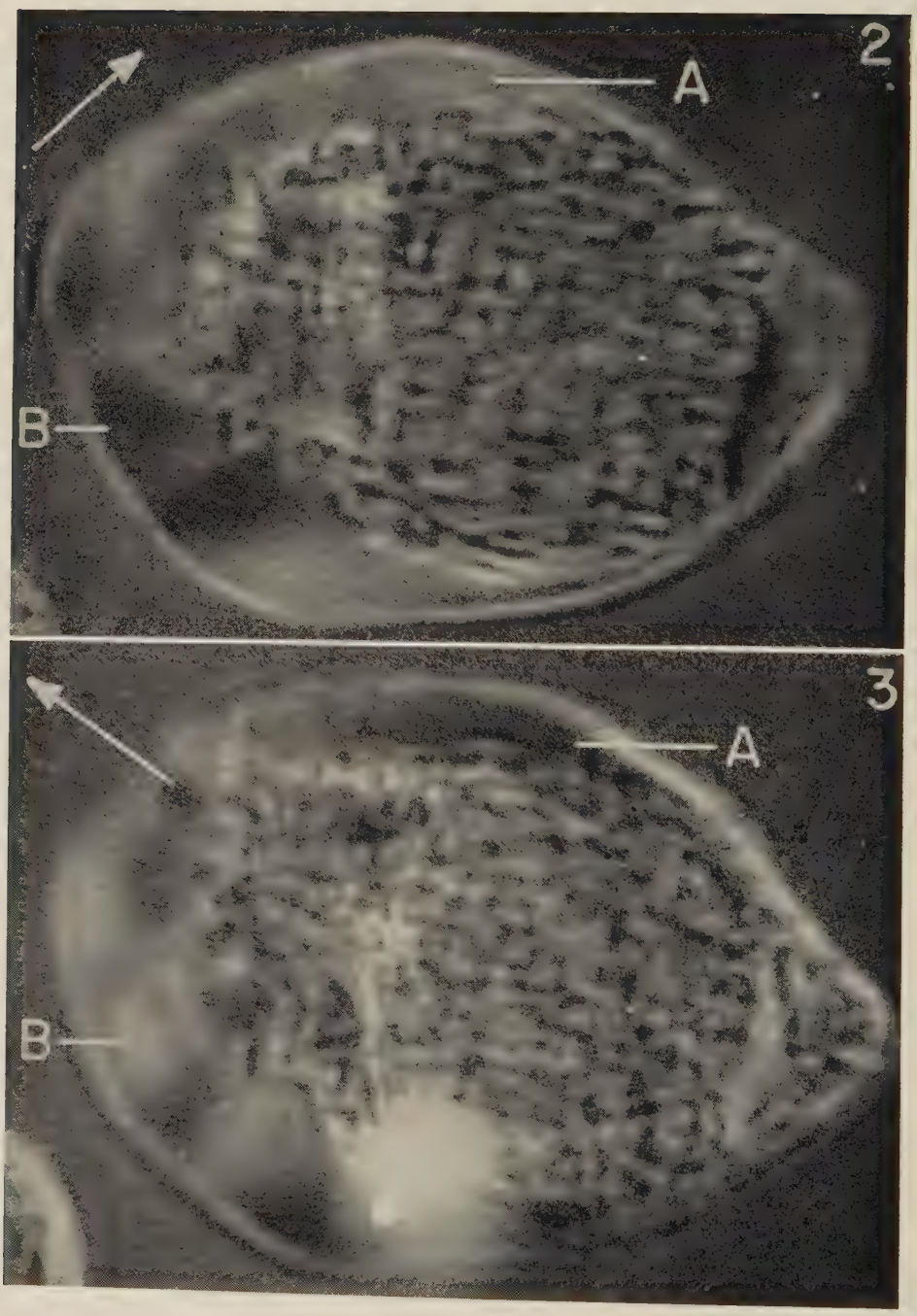


Fig. 1.—Electron micrograph of human plasma cell. Circumferential lamellae of endoplasmic reticulum (*ER*) and the associated dense particles (*P*), mitochondria (*M*), and small vesicles which probably belong to a Golgi system (*gs*) may be seen in the cytoplasm. An excentrically located nucleus is seen at *N*. × 7500.

contrast (*A*, *B*). A comparison between the cell in Fig. 1 and the cell in Figs. 2 and 3 reveals a similar distribution of areas containing endoplasmic reticulum and areas of birefringence. The irregular course of the membranes may be expected to produce variations in the axis of birefringence such as those observed at *A* and *B* in Figs. 2 and 3.

The simplest interpretation of the radially positive birefringence in this case is that it is the algebraic sum of a form component, which for the circumferentially oriented lamellae would be negative, and a larger positive intrinsic component. Positive form birefringence would also be produced by radially oriented rods, but morphological evidence at hand provides no support for such an alternative assumption. A positive intrinsic birefringence suggests, among other things, that there is contained in the membranes which are seen in electron micrographs a layer of radially oriented lipid molecules and that lipoprotein models which have been proposed for the cell membrane, myelin, etc. may also be applicable to endoplasmic reticulum.

We conclude from this study that the endoplasmic reticulum seen in human plasma cells with the electron microscope is present in similar arrangement in the living cell. In this restricted sense the fidelity with which structure is preserved by osmic acid fixation and methacrylate embedding is corroborated.


2
A
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3
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TECHNICAL IMPROVEMENTS IN SPECIMEN PREPARATION FOR ELECTRON MICROSCOPY

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The securing of high resolution electron microscopic pictures depends not only on mechanical and electric factors concerning the microscope utilized, but on several factors related to the material examined, particularly when dealing with biological material. This is well-known to electron microscopists who have repeatedly tried to improve conditions of the material to be prepared for examination.

The high level attained by ultramicrotomy is keeping pace with the resolution obtainable with the electron microscope. Attention was directed by some workers to improving the quality of supporting films in order to eliminate drifting of the specimen (carbon films of Watson [2]) or even to suppress the film itself, in order to increase the contrast of high resolution "in focus" pictures (Formvar nets of Sjöstrand [1], carbon holes of Watson [2]).

The thickness of the sections submitted to the electron beam is a source of electron scattering and consequent drop of resolution. When obtaining very thin sections (about 200 Å) inconveniences resulting from compression are always present even in

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Figs. 2 and 3.—Photomicrographs of a living human plasma cell taken with the polarizing microscope. The direction of the electric vector of the polarizer is indicated by the arrow. The compensator was set to give maximum photographic contrast of birefringent areas such as *A* and *B*. Note the reversal in contrast in these areas when the cell is rotated 90° by comparing Figs. 2 and 3. × 7500.

the best cutting conditions. Compression brings about, to varying degrees, shape modifications on biological structures and artificially increases the thickness of the sections.

The present note is designed to recommend a flattening procedure for ultrathin sections currently used in our laboratory and to stress the importance of carbon films for obtaining a grade of specimen stability compatible with high resolution electron microscopy.

The flattening procedure is based on the action of chloroform on polymerized methacrylate, and carbon films are obtained by the same method utilized by Watson, but in a simplified way.

Under unfavorable conditions, a considerable degree of compression develops, but if several influencing facts such as size of the knife-edge angle, size of the trimmed face of the block and hardness of the embedding material are settled in an adequate way, compression may be brought down to a minimum. A mixture of water and acetone (or water–alcohol) is commonly used to receive the sections, and a short permanence in this mixture contributes to their flattening. However, by comparing the size of the block face with the size of the sections it can be easily demonstrated that total expansion rarely occurs.

The electron microscopic examination of such sections may not reveal great distortion of cell structures but elongation of the profiles of circular structures is frequently seen.

It was found that when a small amount of chloroform vapors reaches the water surface in which the sections are floating, these expand almost immediately. The procedure is carried out in a simple and quick way. It is sufficient to pass a tiny piece of filter paper moistened with chloroform, over the ribbon of sections, without touching these. It is then observed that as a result of the expansion obtained, a change of the interference colors develops (gold-silver sections become silver, and silver sections become transparent). The optimum working distance is easily appreciated while observing the expansion process and the worker is able to stop the action of chloroform by moving back the tweezer. It is obvious that the filter paper must not be placed too close to the sections or must not be soaked excessively in chloroform because a large amount of vapors may cause both the breaking of the ribbon and the spreading out of sections.

It was found that the action of chloroform considerably simplifies actual working conditions and that even the best sections are susceptible of some degree of expansion after chloroform treatment.

Chloroform does not alter the quality of the embedded material, provided its action be moderate, i.e., lasting just the time necessary for the expansion to take place. Too lengthy a permanence of chloroform is a handicap, for chloroform is a solvent of polymerized methacrylate.

The sections are obtained with the Porter-Blum microtome (mechanical advance); they are then treated with chloroform and mounted on uncoated grids and allowed to dry as usual. The grids with the sections are then put in the metal evaporator and coated with a thin film of carbon. Examination is carried out with an R.C.A. EMU 2C electron microscope.

It was found that using small space grids a very thin coat of carbon (just the amount to enable the film to become barely visible in a white testing porcelain) is

enough to reinforce the sections so that they can withstand the electron beam. No other supporting films (parlodion or formvar) are, of course, required.

The carbon coat must be slightly thicker when using grids having spaces bigger than those indicated above. When employing this coating procedure care must be taken that sections have as few knife scratches as possible because these seriously impair the specimen stability under the electron beam.

Another shortcoming of this procedure is the relative facility by which the free edges of the ribbon move under the beam, but it was noted that in areas a little apart from the edge section or from a knife-scratch pictures can be taken without loss of resolution.

The simplified procedure allows the suppression of several steps of carbon films preparation and have almost all the advantages indicated by Watson for this type of films, except for the above-mentioned limitations.

Summary.—Two improvements in preparation of specimens for electron microscopy have been described. One of them involves the expansion and flattening of ultrathin sections by means of chloroform vapors. The second improvement consists in mounting the section in grids without supporting film, coating them with a thin layer of carbon.

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THE *IN VITRO* INCORPORATION OF C^{14}-LEUCINE INTO THE PROTEINS OF TUMOR MICROSOMES AND A NOTE ON THE PROPERTIES OF ISOLATED TUMOR MITOCHONDRIA

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Zamecnik and Keller [10] have shown that rat liver homogenates, freed from nuclei and mitochondria and fortified with an ATP-generating system,[1] actively incorporate amino acids into their microsomal proteins. The microsomes of primary rat hepatomas, on the other hand, have very recently been reported by Campbell and Greengard [3] to have largely lost this ability *in vitro*. The latter statement prompts us to

[1] Abbreviations are used as follows: ATP for adenosine triphosphate, DPN for diphosphopyridine nucleotide, *p*-DAB for *p*-dimethylaminoazobenzene.

TABLE I. *In vitro incorporation of* DL-*leucine-1-C*^{14} *into the proteins of subcellular preparations from tumors.*

The data are expressed as per cent of the radioactivity shown by the corresponding fractions of normal liver. For details compare experimental part. The case numbers of the tumors are listed, their nature is mentioned in the text.

Tumor	Extra addition	Radioactivity of proteins
T 5441	—	100
T 5358	—	86
T 28012	—	41
Hepatoma (*p*-DAB, rat)	—	10
T 26473	—	9
	ATP	17
	KF	17
T 26567_S	—	0
	ATP	17
	KF	15
T 17572	—	0
	ATP	0
	KF	0

report on our experiments in which the *in vitro* incorporation of DL-leucine-1-C^{14} into the proteins of the microsomes of a number of tumors and the biochemical behavior of the corresponding tumor mitochondria has been studied.

Experimental. -Liver and tumor tissue was homogenized at 0°C in the medium X (using 2.5 ml/g fresh tissue) of Zamecnik and Keller [10]. Flasks were filled with 0.7 ml of the homogenate (containing from 4 6 mg nitrogen) which was freed from nuclei and mitochondria by centrifugation during 10 minutes at 5000 × *g*.

DL-Leucine-1-C^{14} (33 μg, containing 0.33 μC), 0.05 ml hexose diphosphate (0.2 *M*), 0.05 ml nicotinamide (0.2 *M*) and 0.1 ml DPN (0.016 *M*) were added, and 0.05 ml ATP-K_4 (0.2 *M*) or 0.05 ml KF (0.4 *M*) as indicated; total volume 1.0 ml.

The flasks were stoppered and incubated during 60 minutes at 37°C in air with gentle shaking. At the end of the incubation period, the protein was precipitated with trichloroacetic acid, defatted, washed, dried and counted on 1.1 cm^2 area at "infinite thickness" under a Geiger–Müller end-window counter.

Results and Discussion.—The incorporation of DL-leucine-1-C^{14} into the proteins of the sub-cellular preparations from seven different tumor strains is illustrated in Table I. The data are expressed as per cent of the radioactivity shown by the proteins of the corresponding preparations from normal liver incubated under the same conditions. The counts/min/mg liver protein (mouse, rat) were of approximately the same magnitude as those reported by Zamecnik and Keller [10]. Incorporation took place mainly in the microsome fraction.

In three transplanted mouse tumors—one adrenal cortex carcinoma (T 17572), one sarcomatoid ovarian tumor (T 26567_S) and one hepatoma (T 26473)—and in primary *p*-DAB-induced rat hepatomas no radioactivity could be detected in the proteins. However, positive results were obtained in experiments with a number of other transplanted mouse tumors. The subcellular fractions from a granulosa cell tumor of the ovary (T 5441) and an interstitial cell carcinoma of the testis (T 5358) equalled the liver fractions in their ability to incorporate DL-leucine-1-C^{14}. In the case of another hepatoma (T 28012) moderate labeling of the proteins was observed. Preparations from rat liver, dissected free from adhering hepatoma masses, showed an incorporation of amino acid comparable to that of normal liver. The same was found with livers of rats on the *p*-DAB diet in which no hepatoma had as yet developed.

The failure to effect protein labeling *in vitro* might have been due, among other reasons, to the fact that the ATP-dependent amino acid activation step was impaired in some of the tumor preparations. In view of this possibility it is of interest that the particular tumor microsomes contain not only very active DPNases but also very active ATP-splitting enzymes [8]. The former enzymes, which might abolish the glycolytic reactions by destructing the coenzyme and thus block the necessary ATP-supply, were successfully counteracted by adding nicotinamide. ATP-splitting can be eliminated by adding fluoride. This compound does, however, inhibit glycolysis as well and would for this reason appear to be unsuitable for the present purpose. Addition of KF resulted, in fact, in strong inhibition of the amino acid incorporating system of normal liver. Nevertheless, as shown in Table I, fluoride caused a higher incorporation of C^{14}-leucine into the proteins of T 26473 and T 26567_S. Addition of ATP had the same effect. On the other hand, neither fluoride nor ATP had any effect in the case of T 17572 or the rat hepatomas.

It is of further interest to note that the degree of amino acid incorporation displayed by the microsomes of the various tumors is exactly paralleled by the extent of the functional integrity shown by the sucrose-mitochondria from the corresponding tumors [5, 6, 7]. For example, the mitochondria from T 5441 and T 5338 oxidize fatty acids and pyruvate under certain conditions, show low ATP- and DPN-splitting activities and do not impair the octanoate or DL-β-hydroxybutyrate oxidation of normal mouse liver mitochondria when added to the latter. The mitochondria from the rat hepatomas, T 26567_S, T 17572 and T 26473, show exactly the opposite behavior, T 26473 being the least characteristic example of this group. T 28012 has been classified in a group of tumors whose mitochondria show properties which lie in between those of the two earlier groups (cf. reference [4] for a review of these experiments). The inhibition of octanoate or L-β-hydroxybutyrate oxidation which is observed in the combined system of normal liver mitochondria and mitochondria from a tumor such as T 17572, has been found to be due to the fact that the active ATPases of the latter mitochondria abolish the ATP-dependent fatty acid activation reaction of the liver mitochondria [7]. Similarly, the microsomes of these tumors may impair the amino acid activating step of the soluble fraction by splitting the ATP required in this reaction.

Since the above correlation between the behavior of the mitochondria and the microsomal-soluble fractions of the tumors was found, the possibility was considered that the differences in behavior of the various tumor mitochondria might have been

due to an admixture of microsomes. Although ribose determinations or staining with pyronine Y [9] did not reveal [8] any significant difference among the ribonucleic acid content of the various mitochondrial samples, except for those from the rat hepatomas which showed higher values, yet some indication for the above assumption was found. By applying the medium of Birbeck and Reid [1, 2] for the isolation of the mitochondria – a procedure which is considered by the authors to minimize the risk of microsomal contamination – we have now been able to prepare [8] mitochondria showing low ATP- and DPN-splitting activities from the group of tumors which include T 17572 and the other tumors listed above.

I am grateful to Drs. P. J. Brombacher and G. H. van Vals for their collaboration in the experiments.

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EFFECTS OF ANDROGENS ON THE CYTOLOGY OF X-IRRADIATED SEMINAL VESICLES

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Delay of mitosis following exposure to ionizing radiations has been demonstrated in a great variety of cells. X-irradiation[2] of the secondary sex organs of the castrate male rodent and subsequent stimulation by an androgenic steroid seemed to offer a unique opportunity for a study of this phenomenon in mammalian cells. The resting secondary sex organs (prostate and seminal vesicles) of the castrate rodent react to

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[2] Irradiation was performed by the Radiology Service.

TABLE I. *Mitotic index of the epithelium of castrate male mice treated with colchicine 6½ hours before death.*

Group	No. of mice	Hours after treament with X-rays 3000 r	Hours after treament with TCP 0.3 mg	TCP after X-rays (hours)	Seminal vesicle mitotic index (%)[a]
A	2	48	None	—	0
B	11	None	48	—	11.6 ± 1.4
C	6	48.5	48	0.5	0
D	3	72	48	24	0
E	3	96	48	48	0.7 ± 0.3
F	4	120	48	72	0.5 ± 0.5
G	9	144	48	96	8.1 ± 0.4
H	5	None	72	—	12.0 ± 5.0
I	6	72.5	72	0.5	1.6 ± 0.3

[a] Mean ± standard deviation of the mean.

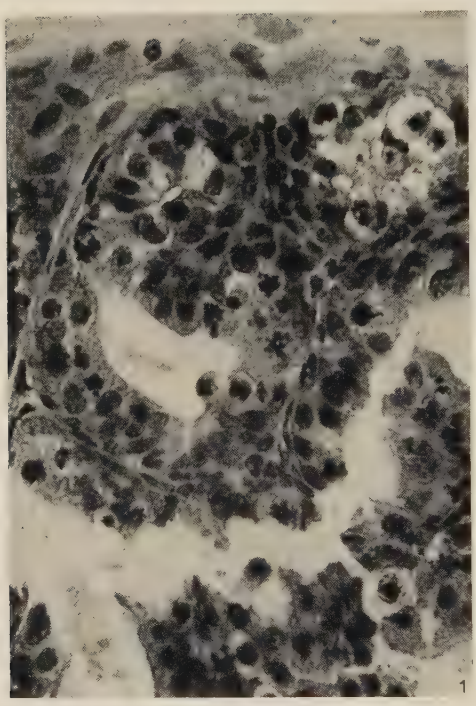
stimulation by an androgen in a thoroughly predictable way by growth and differentiation of the cells and by mitosis. Maximal mitotic activity can thus be induced experimentally. In a previous publication [5] it was shown that X-irradiation of the accessory sex organs of the castrate male rat and subsequent treatment with testosterone propionate results in cellular hypertrophy with delayed onset of mitosis under certain experimental conditions. These findings were recently confirmed [8]. It seemed of interest to study this phenomenon in mice with special reference to the mechanism of recovery from radiation damage.

Male mice of the CF #1 strain were castrated shortly after puberty and were used not earlier than one month after castration. X-irradiation was given through a portal centered over the pelvis with a Philips Contact Therapy Unit (50 KVP, 1.5 MA at 2 cm distance, HVL 0.2 AL; 1045 r/min) using a circular cone of 1.5 cm diameter. During irradiation the mice were under anesthesia with amobarbital sodium (12 mg/100 g bodyweight).

Testosterone cyclopentylpropionate (Depo-Testosterone, Upjohn) (TCP) dissolved in cottonseed oil was given in a single dose of 0.3 mg/100 g body weight intramuscularly.

Colchicine (0.3 mg/100 g body weight) was administered in an aqueous solution (1:1000) intraperitoneally 6½ hours before sacrificing the mice with ether. The seminal vesicles were fixed in 10 per cent formalin and stained with hematoxylin–eosin.

In another series of experiments no colchicine was given. The seminal vesicles were fixed with acetic alcohol formalin. Toluidine blue was used to demonstrate basophilia. Cytoplasmic basophilia was identified histochemically as ribonucleic acid (RNA) by extraction with 2 *M* HCl at 3°C for 16 hours by the technique of Fisher [2]. The sections treated by this procedure were indistinguishable from those treated with crystalline ribonuclease (1:10,000) for two hours at 37°C at pH 5.5.



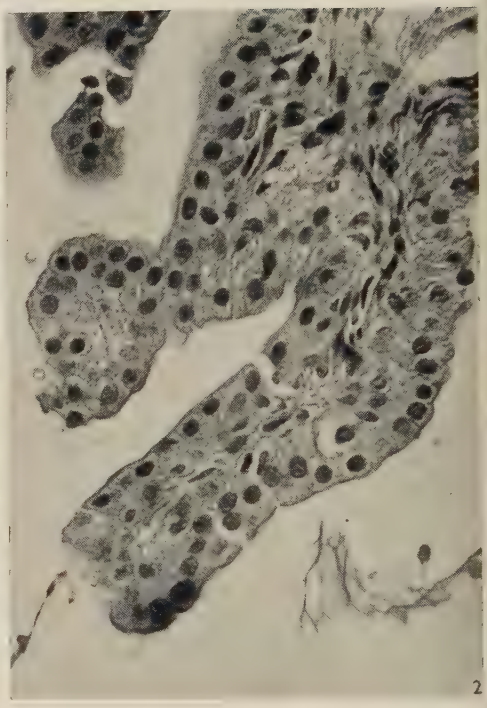


Fig. 1.—Seminal vesicle of castrate mouse. 3 days after treatment with TCP. Colchicine 6½ hours before death. Hematoxylin–eosin, × 255.
Fig. 2.—Seminal vesicle of castrate mouse. 3 days after irradiation and treatment with TCP. Colchicine 6½ hours before death. Hematoxylin–eosin, × 255.

The results on the effect of X-irradiation and TCP on mitosis are summarized in Table I. Irradiation with 3000 r inhibits mitosis completely or nearly completely if the androgen is injected from 30 minutes to 72 hours after X-irradiation and the mice are killed 48 hours later. If 96 hours are allowed to elapse between irradiation and administration of TCP (Group G) recovery of the mitotic activity is complete or nearly complete. The difference in mitotic activity between Group B (TCP only) and Group G is not significant statistically ($P > 0.5$). If the time interval between irradiation and administration of TCP is extended to 120 hours, irradiation changes of an inflammatory nature prevent an accurate count of mitoses.

In seminal vesicles of animals receiving TCP 30 minutes after irradiation and sacrificed 72 hours later (Group I) mitotic activity is still very low. The difference between Group I and Group H (TCP only for 72 hours) is significant ($P < 0.01$).

Mitosis seemed to be delayed specifically; the irradiated seminal vesicles showed response to the androgen by transition from the cuboidal type of resting epithelium to the secretory columnar type. This transition was indistinguishable from that observed in mice that had received TCP only.

In terms of histochemistry the absence of mitotic figures in the irradiated specimens indicates interference with the formation of chromosomal material, presumably deoxyribonucleic acid. On the other hand X-irradiation seems not to inhibit the formation of ribonucleic acid (RNA), under the conditions of the experiment. Intense cytoplasmic basophilia was found both in non-irradiated and irradiated seminal vesicles of mice treated with TCP. That this basophilia is due to the presence of RNA can be shown by the histochemical methods mentioned above. Furthermore, it has been found by chemical analysis that testosterone propionate increases the amount of RNA phosphorus of seminal vesicles of castrate rats [4, 9]. The increase in the RNA content of the epithelial cells of the seminal vesicles can be correlated with their activity in protein synthesis [4, 7, 9]. The increase in weight induced by testosterone in accessory sex organs of castrate male rats is only partially inhibited by X-rays [3, 8]. In this connection the large nucleoli in the epithelial cells of the irradiated specimens should be noted (Fig. 2); the importance of this structure for the secretory activity of the epithelial cells has been suggested [7].

The data on recovery show that the inhibition of mitosis produced by irradiation diminishes with the increase of the time interval between irradiation and administration of the growth stimulus, e.g., testosterone. The findings in mammals are comparable to the results of experiments on echinoderms and on protozoans. Unfertilized eggs of the sea urchin, *Arbacia punctulata* were irradiated and subsequently fertilized. X-irradiation produced a delay in first cleavage of the ovum and this delay was found to diminish with increase of the time interval between X-irradiation and fertilization [6]. Similar results were obtained in *Pelomyxa carolinensis*. In this large amoeboid organism the nuclei undergo mitosis synchronously. A pronounced delay is produced in the first division following X-irradiation during interphase. The delay in the second division is much smaller than that in the first division and later divisions show complete recovery [1].

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COMPARATIVE MASS DETERMINATIONS OF ISOLATED LIVER CELL NUCLEI BY SCHLIEREN AND INTERFERENCE MICROSCOPY

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Mass determinations have recently become increasingly important for many histochemical and cytochemical studies. Determinations of the mass of a cell or of a cell constituent can yield information on chromosomal and other nuclear changes related to processes of growth and malignancy. Several methods are available for this purpose: X-ray absorption, interference microscopy, and schlieren microscopy. Interference microscopy is a convenient, and both theoretically and empirically well established way to determine the anhydrous mass of a cell. The investigations reported here were performed with the Smith type interference microscope [11, 12], which is basically an interferometer system, with two double-refracting crystalline plates as beam dividers which split and recombine the light beam. The results obtained were compared with those obtained by another optical device [7] which can be called a schlieren system. The present report deals with a comparison of these two approaches.

The mass determinations were performed on rat liver cell nuclei isolated by centrifugation in sucrose and resuspended in 70 per cent alcohol. Several slides were prepared from this suspension. The interferometric measurements were made visually, using an AO-Baker interference microscope. The rotatable analyzer of this instrument has been set, first, to provide minimum illumination of the background adjacent to a particular liver cell nucleus. The analyzer was then turned until minimum illumination was obtained for the center of the nucleus. From the difference in the readings on the analyzer scale and by knowing the size of the specimen, assumed to be spherical, its index of refraction and dry mass were computed. The proper procedure followed the details given by Barer [1, 2], Davies [4, 5], and Mellors [6]. Twenty-five liver cell nuclei on four slides have been measured in this manner.

Nuclei on these same slides were then analyzed by schlieren technique. Schlieren are optical inhomogeneities which direct light passing through into directions other than the "normal direction". That deviation depends upon local differences, Δ_n, between the refractive indices of the specimen and its surroundings and upon the shape of the detail. The optical arrangement used here has been briefly described in [8]. The theory involved in optical systems being comparable to this is rather complicated; details may be found in comprehensive articles by Schardin [10] and Wolter [13].

Any standard microscope can be converted into a schlieren microscope, simply by inserting a grating approximately half-way between the back focal plane of the objective and the eyepiece. No special objectives or coverslips are needed. As in interference microscopy, the image seen through such a schlieren system has super-

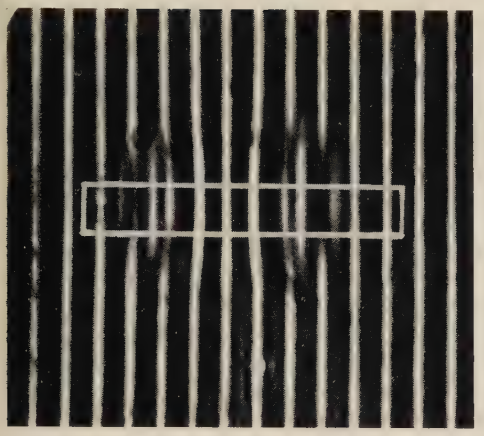

Fig. 1.

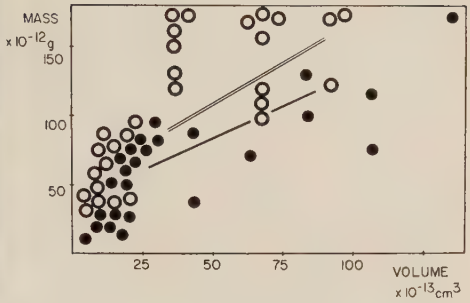

Fig. 2.

Fig. 1.—Schlieren photomicrograph of an isolated liver cell nucleus. Magnification × 1400. The area across the nucleus indicates the tracing by the microdensitometer.
Fig. 2.—Comparison between mass determinations of isolated liver cell nuclei by means of an interference microscope (• • •) and the schlieren microscope (○○○).

imposed upon it a pattern of alternating black and white stripes. From disfigurations and displacements of these stripes several refractive properties of the sample can be calculated. Fig. 1 shows an example of a schlieren photomicrograph taken from one of the isolated liver cell nuclei. Some displacements of the background pattern are clearly visible. Twenty-nine measurements were made by this technique.

The displacement of any line in a schlieren photograph may be designated D, the distance between the geometrical center of a cell nucleus and the displaced part of this line, p, and the specific refractive increment, α. The dry mass concentration, expressed in grams per 100 ml of solution, can then be given in a first approximation as

$$C \simeq n_M \cdot \frac{D}{p} \cdot \frac{1}{\alpha}, \tag{1}$$

provided the refractive index of the cell is greater than the refractive index, n_M, of the mounting medium.

The results of our measurements are shown in Fig. 2. The volumes of the isolated liver cell nuclei (in units of 10^{-13} cm^3) have been plotted against their total dry mass ($\times 10^{-12}$ g) as determined by both interference and schlieren microscopy. In general, the figures obtained by schlieren microscopy are slightly higher than those by interferometry. This aberration may be due, in part, to curved surfaces of the cell-object. Another error may be introduced by the fact that the specific refractive increment, α, cannot be applied, in general, to substances being in a separate phase from the liquid. However, this would apply to both our interferometric and schlieren measurements.

Other investigations were carried out on living yeast cells (immersed in water) and on ascites carcinoma cells and erythrocytes (immersed in ascites fluid [9]). They seem to indicate that this deviation may represent a systematic error. The dry mass of an erythrocyte, for instance, has been estimated as 36×10^{-12} grams by schlieren technique [9], compared with 31.4×10^{-12} grams by interferometry [1]. Finally, it may be noted that the two types of interference microscopes available on the market, the Dyson and the AO-Baker instrument, are said to give not always identical results [3]. The resaon for that discrepancy has not been found. In spite of the potential deviations mentioned above and despite the fact that schlieren microscopy is, for optical reasons, less sensitive than interference microscopy, it is interesting to note that comparable results were obtained. The schlieren system described can simply be built as an attachment to any eyepiece which can then convert any standard microscope into a schlieren microscope. Such a device may thus serve as an adjunct to present methods of local refractometry.

Summary.—The dry mass of isolated liver cell nuclei was determined with an interference microscope and with a schlieren grating microscope. Sufficient agreement between the results obtained indicate that the latter new, inexpensive instrument provides a means of measuring refractive properties of cells or cell structures.

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CATABOLISM OF GLYCINE-C^{14} BY WASHED BOVINE SPERMATOZOA[1]

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Evidence has been presented [3] indicating that bovine spermatozoa metabolize glycine-1,2-C^{14}, but the use of the uniformly labeled amino acid did not permit determination of the fate of individual carbon atoms.

In the present report CO_2 and formate production from both glycine-1-C^{14} and glycine-2-C^{14} have been studied; the requirements of the system for oxygen and the effects of certain potential cofactors and inhibitors also have been determined.

Materials and Methods.—The spermatozoa used in these trials were obtained and prepared for use as previously described [2]. Spermatozoa thus prepared and suspended in Ringer-phosphate buffer were used in isotopic or manometric studies within 2 hours after collection.

Experiments were conducted in Warburg flasks with alkali and filter paper in the center well. Each flask contained 10^9 spermatozoa in a final volume of 2 ml. Manometric studies were conducted by conventional methods [11] at 37°C with air as the gas phase. The glycine concentration was maintained at 0.01 *M*; when formate was to be recovered, 0.01 *M* sodium formate was included to serve as a metabolic trap.

At the end of the incubation period, 10 *N* sulfuric acid was tipped into the flask to stop the reaction and an additional 15 minutes was allowed for CO_2 absorption. Contents and washings from the center well were transferred to a centrifuge tube and barium carbonate samples prepared as previously described [4]. When formate was to be assayed, contents of the main flask were deproteinized, centrifuged, and the supernate steam distilled at pH 3.0 [1]. The distillate was reduced in volume under alkaline conditions, then the formate was oxidized selectively with HgO [9]. CO_2 from formate oxidation was trapped in a sodium hydroxide tower and converted to barium carbonate for radioassay. Radioactivity was measured in a windowless gas flow counter; counting cycles were of sufficient length to reduce the counting error to 3 per cent or less.

Results and Discussion.—Glycine-1-C^{14} and glycine-2-C^{14} were employed to determine the fate of the carbon atoms of glycine during catabolism by washed bovine spermatozoa. Results of 3 such trials in which CO_2 and formate were recovered are presented in Table I. In each trial the carboxyl carbon was converted to CO_2 more rapidly than was the α-carbon. Similarly more formate arose from the α-carbon

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TABLE I. *Production of labeled CO_2 and formate from glycines 1- and 2-C^{14} during 2 hours incubation at 37°C.*

Activity added μc	Recovered in products from -1-C^{14} μc	-2-C^{14} μc	Products from glycine-1-C^{14} CO_2	Formate	Products from glycine-2-C^{14} CO_2	Formate
			cpm per μmole of product per μc activity added			
0.4	0.003	0.0004	252	0.26	107	0.45
0.5	0.005	0.002	262	2.32	93	3.46
2.0	0.018	0.007	241	4.70	85	6.00

than from the carboxyl carbon. The formate specific activity was much lower than that of CO_2, although the total activity recovered in formate often approached that recovered in CO_2. Although formate occurs in freshly collected bovine semen [5], its metabolic role and rate of turnover have not been explored. Obviously within a 2-hour incubation period some of the formate produced from glycine could have been further oxidized to CO_2. This being the case, one would expect the ratio (CO_2 from glycine-1-C^{14})/(CO_2 from glycine-2-C^{14}) to become smaller as the incubation period is lengthened.

When incubation periods of 30, 60 and 120 minutes were compared (Table II), a tendency for this ratio to decrease with longer incubation was noted when total radioactivity in CO_2 was considered. A second trial in which incubation periods of 10 and 60 minutes were used provided more obvious differences in ratios. Such data suggest a more direct oxidation of the carboxyl carbon than of the α-carbon. It also demonstrates the speed of the reactions, for 32 and 46 per cent (α- and carboxyl-carbons, respectively) of the radioactivity present in CO_2 during 2 hours was released in the first 10 minutes of incubation. The relative rate of production of CO_2 from the carboxyl and α-carbons of glycine, as shown in Tables I and II, is in agreement with the results of Nakada and Weinhouse on liver slices [8].

Although glycine is utilized by animal tissues, it is often difficult to demonstrate either oxygen uptake or ammonia liberation when glycine is used as the substrate [7]. In this respect spermatozoa do not differ from other animal cells; Tosic and Walton [10] reported no ammonia liberation from glycine, and a series of trials in this laboratory have consistently shown little or no increase above the endogenous level of oxygen uptake when glycine (0.01 *M* in Ringer-phosphate, pH 7.4) was the substrate for washed bovine spermatozoa. Since appreciable oxygen uptake was not observed, it was not feasible to measure the ratio of oxygen uptake to glycine disappearing.

Various potential cofactors and inhibitors were used in efforts to alter the oxygen uptake and thus perhaps provide an indication of the mechanism of glycine catabolism. These studies included 0.001 *M* adenosine triphosphate, 0.015 *M* Mg^{++}, 0.02 *M* fluoride and 0.0001 *M* 2,4-dinitrophenol; no significant alteration of oxygen uptake was observed in 2 hours incubation at 37°C.

In manometric studies, it was observed that oxygen uptake with glycine in Ringer-phosphate often barely exceeded the endogenous respiration for the first 30 minutes

TABLE II. *Rate of incorporation of C^{14} into CO_2 from glycines 1- and 2-C^{14}.*

Time of incubation (min)	Total activity of CO_2 derived from glycine			Specific activity of CO_2 derived from glycine		
	-1-C^{14} (cpm)	-2-C^{14} (cpm)	Ratio 1-C^{14}/2-C^{14}	-1-C^{14} (cpm/μM)	-2-C^{14} (cpm/μM)	Ratio 1-C^{14}/2-C^{14}
Expt. 1						
30	5350	1550	3.5	340	125	2.7
60	6440	2070	3.1	340	115	2.9
120	6600	2340	2.8	335	118	2.8
Expt. 2						
10	3020	740	4.1	244	59	4.1
60	6260	2060	3.0	365	139	2.6

of incubation, then dropped to or below the endogenous level. Semen is known to be low in catalase [10], and since it has been proposed that the oxidation of formate to CO_2 is catalase-linked [8], the effects of catalase on glycine oxidation were tested. Beef liver catalase (1.4 units/ml sperm suspension) appeared to maintain oxygen uptake slightly above the endogenous level, but this effect was not dependent on the presence of glycine. Reports indicate that spermatozoan motility during storage is increased by the addition of catalase to egg yolk-citrate and milk diluents [6, 12].

The dependence of the system on the presence of oxygen was determined by comparing the production of CO_2 from glycine-1-C^{14} with air as the gas phase and with nitrogen as the gas phase. As shown in Table III, exclusion of oxygen in the absence of glucose resulted in a 20 per cent reduction in the yield of $C^{14}O_2$. Aerobically the addition of glucose did not appreciably alter the yield of $C^{14}O_2$ although CO_2 from glucose caused a reduction in the specific activity of 30 per cent. Anaerobically glucose addition resulted in a reduction, both in yield and specific activity, of about 70 per cent. Thus under anaerobic conditions glycine is metabolized slowly so long as glycolysable substrate is present; in the absence of such substrate glycine is readily utilized. Although extensive studies of glycolate metabolism have not been conducted, evidence has been obtained that aerobically with washed cells, the yield of $C^{14}O_2$ from glycolate-1-C^{14} is about 85 per cent of that from glycine-1-C^{14} while the yield of labeled glyoxylate is much greater from glycolate than from glycine. In contrast to glycine, utilization of glycolate is dependent to a large extent on the presence of oxygen.

Conversion to serine is considered to be an important pathway of glycine metabolism in many tissues [7, 8]. Repeated attempts to demonstrate such conversion by bovine spermatozoa have not been successful, and it appears that the amount of serine formed from glycine-C^{14}, if any, is extraordinarily small compared to the amount formed in other animal tissues.

TABLE III. *Effect of oxygen and glycose on the production of $C^{14}O_2$ from glycine-1-C^{14}.*

Gas phase ...	Air		Nitrogen	
$C^{14}O_2$ ...	Yield (cpm)	Sp. act. (cpm/μM)	Yield (cpm)	Sp. act. (cpm/μM)
No glucose				
Expt. 3	3490	250	3170	236
4	3200	256	2860	310
5	3160	216	1740	180
Average	3283	241	2590	242
Glucose added				
Expt. 3	3290	172	900	102
4	3180	134	800	101
5	3850	168	360	35
Average	3440	158	687	79

These results indicate that the principal pathway of glycine degradation involves glyoxylate, formate and CO_2, but do not exclude the possibility of other pathways. The labeling of these products, the accumulation of much more glyoxylate-C^{14} from glycine, and the apparent limited oxygen uptake and ammonia liberation with glycine may be indicative of transamination. This study is being continued to determine to what extent transamination is involved in glycine degradation by these cells.

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DYE UPTAKE AND SURVIVAL STUDIES IN X-IRRADIATED YEAST CELLS

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A BETTER understanding of the effects of ionizing radiation on plant and animal tissue has stimulated a search for other methods to distinguish the intact living cell from that damaged or killed by ionizing radiation. At present, a method generally used for obtaining dose-effect curves consists of measuring per cent cell survival by the agar-plating technique. This method is time-consuming and also has the disadvantage of revealing nothing of the early radiation effects that lead to cell death. Another method used to determine damage or death of a cell is based on the assumption that viable cells are impermeable to certain dyes while damaged or dead cells will readily stain. Acridine orange has been employed successfully to detect radiation damage in the epidermis of *Allium cepa* [5, 8]. King *et al.* [3] used trypan blue as an indicator of radiation damage in Ehrlich ascites tumor cells. Eosin blue was used by Kereiakes, Hodgson, and Krebs [2] to measure damage to yeast cells exposed to large doses of ultraviolet light.

The present study was initiated in an attempt to evaluate results obtained by the colony-plating and the dye uptake methods when they are employed to yield information relative to damage or death in x-irradiated yeast cells.

Materials and methods.—The yeast used throughout this study was a diploid strain of *Saccharomyces cerevisiae* originally obtained from the American Type Culture Collection and listed as No. 4125. The culture was maintained on and harvested from Difco Potato Dextrose Agar prepared according to specifications. For x-irradiation two day old cells were washed twice and suspended in phosphate buffer, pH 5.6. A suspension containing approximately 70 million cells per ml resulted. Two ml samples of suspension contained in a Carrel flask were irradiated with a North American Philips Unit employing a Machlett AEG-50-T x-ray tube and operated at 50 kvp, 40 ma, and 1 mm Be inherent filtration. The dose rate at a tube-target and suspension-surface distance of 6.0 cm was 18,750 r/min (measured in air with a Victoreen nylon chamber).

Immediately following the radiation exposure 0.75 ml of cell suspension was placed in tubes containing 0.75 ml of aqueous dye solution. The molarity of the dyes varied; however, each registered an initial per cent light transmission of 10 to 30 per cent. All light transmission readings were taken with a Coleman Spectrophotometer with the wavelength setting giving maximum light absorption for that particular dye used. After 35 min. the cell dye suspension was centrifuged and the per cent transmission of light through the supernatant measured. The 35 min. time interval was found to be optimum for maximum dye uptake by heat-treated and by x-irradiated yeast cells.

The routine bacteriological plating technique was used to determine the number of yeast cells surviving the radiation exposure. One-tenth ml samples of irradiated suspension were diluted in sterile distilled water blanks and plated on Potato Dextrose Agar (Difco). After 48 hr incubation at 30°C the colonies were counted and tabulated.

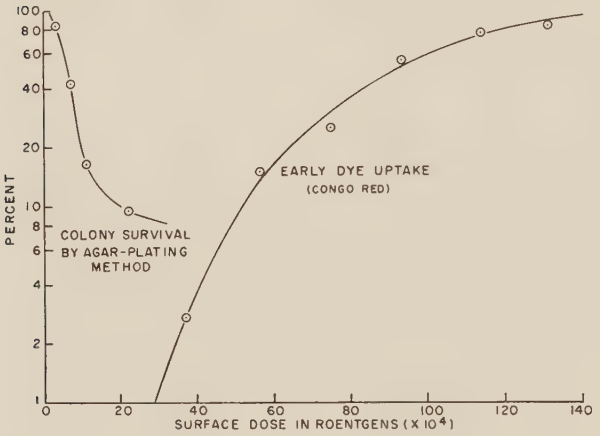

Fig. 1.—Survival–early dye uptake of x-irradiated yeast cells for various surface doses.

Results and discussion. - The data plotted in Fig. 1 represents the results typical of 8 experiments using congo red as the vital dye. In preliminary less extensive studies with evans blue, methylene blue, janus green, eosin B, aniline A, azure A, trypan red, victoria blue, and bismarck brown, similar results were observed. Two experiments with congo red where cells were suspended and irradiated in phosphate buffer at pH 7.0 also showed similar results.

From Fig. 1 it is evident that a total dose of 187,500 r results in a colony survival of less than 10 per cent. At this same total dose the yeast cells exhibited no early uptake of dye when measured spectrophotometrically (500 mμ) or observed microscopically. However, early dye uptake did occur when a two-fold dose increase over that dose necessary to produce 90 per cent death was administered. Once early cell staining was observed, further increases in dose resulted in increasing dye uptake by the suspension until a plateau was reached. No dye uptake was found in parallel studies, for normal unirradiated yeast cells. It is thus apparent that large doses of ionizing radiations, far in excess of those needed to produce death, are required before early staining of irradiated yeast cells with vital dyes occurs. These observations are presented in view of earlier work concerning vital staining of yeasts killed by heat. Knaysi [4] showed that in neutral or moderately acid media, even a slight staining of cell protoplasm in heat-killed yeast and bacterial cells is an evidence of death. Kurusz [6] obtained excellent correlation between the colony-plating method and the staining technique when employing congo red to stain heat-killed yeast cells.

The above data are considered evidence that care should be taken in evaluating studies in which early staining of yeast cells with vital dyes is used as a criteria of viability. Several explanations may present themselves as possible approaches to

this problem. According to Werkman and Wilson [9] the distinction between living and dead cells by vital staining with basic dyes depends on increased permeability on cell death and/or negative staining on a partial autolysis. King *et al.* [3] found that very marked changes in Na, K, and cell H_2O content occurred before trypan blue was taken up by irradiated Ehrlich ascites tumor cells. Lepeschkin [7] observed that in cell death in *Spirogyra* and *Elodea*, dye absorption took place during and after cell-protein coagulation. In the studies presented here it was found that high doses of radiation are required before early dye uptake by cells is observed following radiation exposure. The results suggest that in early x-irradiation damage marked changes in cell permeability are not reflected immediately. The cells may continue some metabolic activity which is largely manifested by non-selective increases in non-aqueous cell constituents and the formation of doublets. However, due to damage to other vital processes, the cells are in a state of degradation and will gradually lyse [1].

Cells thus affected will not produce visible colonies when plated on agar under suitable conditions. These cells are presumed dead and will be recorded as such. On the other hand, cells which follow this pattern will not take up vital dyes soon after radiation exposure and when measured by this method will be considered viable. When exposed to massive doses of ionizing radiation the dye uptake pattern seems similar to that of heat-killed cells. It appears that these large doses of irradiation produce early structural changes in cell protein analogous to those produced by exposure to heat.

Summary.—No apparent correlation was found in parallel studies on the effects of x-irradiation on yeast cells using the early dye uptake and colony-plating methods as criteria. A two-fold increase in dose above that dose required for 90 per cent death was necessary to obtain early dye uptake of congo red by the cells. In view of earlier work on vital dye uptake of yeast cells killed by heat, the results here indicate that for doses of ionizing radiation in the lethal range, early cell permeability and protein structural changes are not pronounced.

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THE ARREST OF MITOSIS AND STOMATOGENESIS DURING TEMPERATURE-INDUCTION OF SYNCHRONOUS DIVISION IN *TETRAHYMENA PYRIFORMIS*, MATING TYPE 1, VARIETY 1[1]

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A method for the synchronization of cell division of the ciliate *Tetrahymena pyriformis* GL was worked out by Scherbaum and Zeuthen previously [7]. Since the organisms of this strain possess only a macronucleus (which divides amitotically), but no micronucleus, we found it desirable to establish synchrony of cell division in a mating strain of *Tetrahymena*. It is obvious that this system offers new possibilities, such as the study of the mitotic division of the micronucleus and the mating reaction at different times in the cell-cycle.

The synchronization of cell division in a mating strain of *Tetrahymena pyriformis* (mating type 1, variety 1)[3] has been achieved by application of principally the same technique as reported earlier [7]. However, the number of the temperature cycles was reduced from 8 to 3 5 and the temperatures of incubation were adapted to this strain. Five alternate ½ hour exposures to temperatures of 42.8 ± 0.2°C and 35 ± 0.4°C were found to induce synchronous division of approximately 80 per cent of the ciliates in a treated population. Sixty per cent synchrony has been obtained with 3 temperature cycles. Dividing ciliates appeared 50 60 min. after the end of the last ½ hour-period at the higher temperature. Division of individuals was noted to require approximately 15 min. from the first appearance of a cleavage furrow.

Ciliates used in these experiments were grown in a medium containing proteose-peptone, liver extract L, mineral salts [8] and glucose (0.5 per cent W/V), at 35 ± 0.4°C, under conditions where oxygen was not a limiting factor for growth; cultures were shaken during incubation or grown in shallow layers of medium in large flasks. Under such conditions the generation time was approximately 1 hr. 50 min. Early stationary phase organisms were used since they were morphologically uniform, had ceased dividing, and yielded a high degree of synchronous division when treated. Ciliates were transferred to fresh medium from such cultures and exposed to the cyclic heat treatment in 250 ml Erlenmeyer flasks containing 50 80 ml of medium. The number of organisms transferred was adjusted to yield a final population density of approximately 50,000 ciliates per ml.

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[3] Synonymous with *T. pyriformis* WH-6.

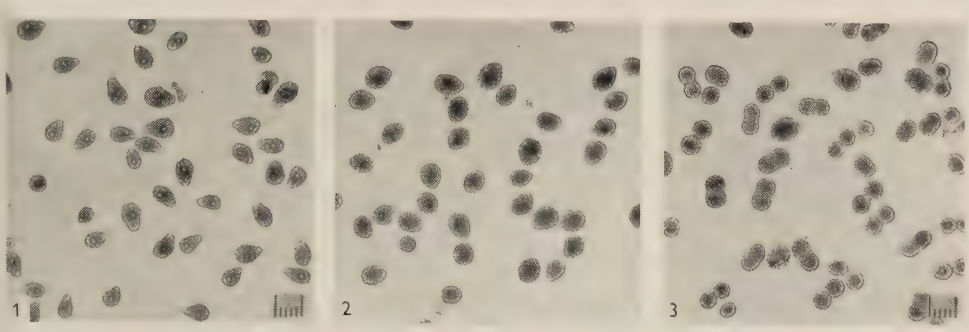

Fig. 1.—Early stationary phase cells (grown at 35 ± 0.4°C), which have just been transferred to fresh medium, shown prior to treatment.
Fig. 2.—Cells after exposure to three temperature cycles (each cycle: ½ hour at 42.8 ± 0.2°C and ½ hour at 35 ± 0.4°C).
Fig. 3.—Cells during first simultaneous division (60 per cent synchrony), 55 minutes after the end of the last high temperature period. The cells were fixed in Bouin's fluid [4]. The distance between two lines on the scales is 10 μ.

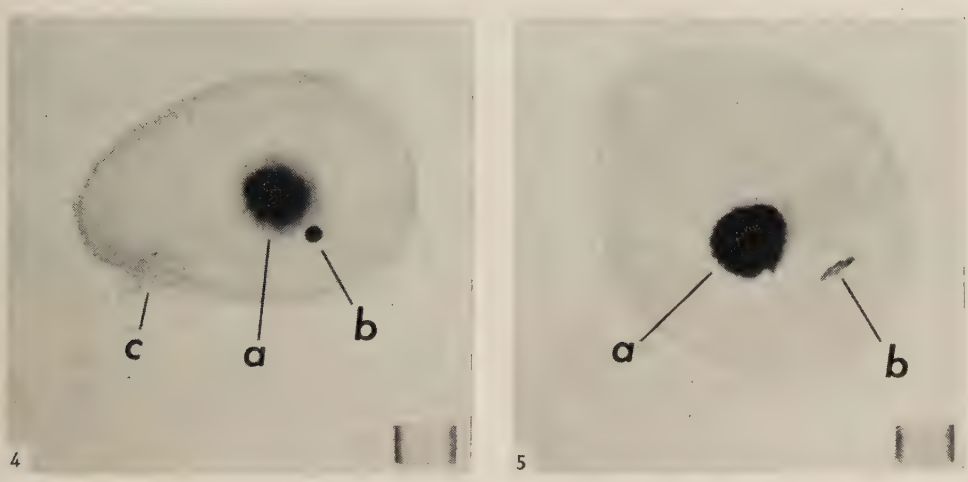

Fig. 4.—A Feulgen-stained preparation of a normal cell. (*a*) macronucleus, (*b*) micronucleus, (*c*) mouth.
Fig. 5.—A cell after application of 4 temperature cycles, stained as cell in Fig. 4. (*a*) macronucleus, (*b*) micronucleus, arrested in anaphase-like configuration. The distance between two lines on the scales is 10 μ.

Changes in the nuclear apparatus, infraciliature and general appearance of the ciliates during the synchronization procedure were followed by Feulgen-staining (Dippell and Chao's modification of the DeLamater stain; Sonneborn [9]), silver impregnation (Chatton-Lwoff technique; Corliss [2]), and the observation of living individuals by phase contrast and interference microscopy.

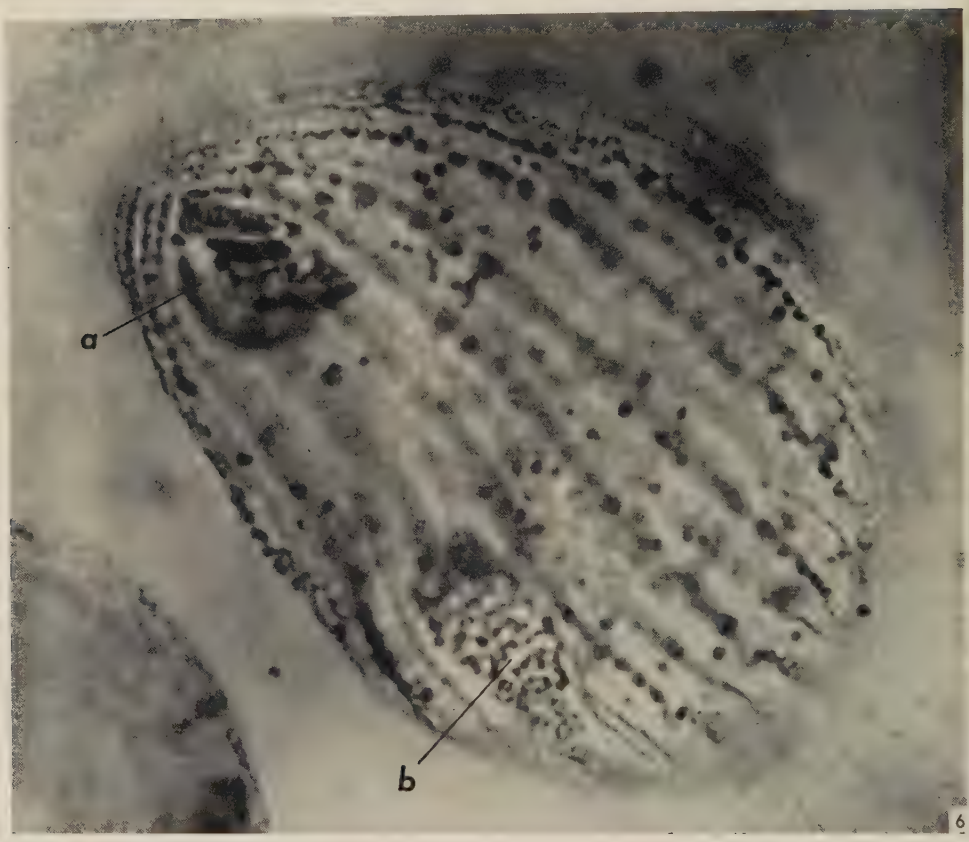

Fig. 6.—A silver-impregnated cell after application of 4 temperature cycles. (*a*) the original mouth of the individual, (*b*) anarchic field, showing the arrangement of kinetosomes of the anlagen prior to organization of functional mouth parts. The distance between the two lines on the scale is 10 μ.

The alternate periods of high and low temperature were found to have the same effects on this strain of *Tetrahymena* as those described by Scherbaum [4] for strain GL (amicronucleate); cessation of division and a gradual increase in volume and mass. Cytological examination of ciliates removed from the culture after each high temperature period disclosed an increasing percentage of organisms in the same general stage of preparation for cell division; the anaphase stage of division of the micronucleus and the "anarchic field" stage of stomatogenesis [1]. These same ciliates displayed a slight notch immediately anterior to the area of stomatogenesis, which was later seen to be at the plane of bisection of the cleavage furrow. At the end of the 5th high temperature period 60–80 per cent of the population sampled was at this early stage of the division process. No further change in the morphology of the nuclear apparatus or infraciliature was observed for 40–50 min. Micronuclear division

and stomatogenesis were then resumed and cell division was rapidly completed (15 min.).

The ability to mate was tested after synchronization, to determine the "normality" of the heat-treated ciliates. Mating type 2 of variety 1[1] was prepared for conjugation [3] 3–4 hours prior to the completion of synchronization of mating type 1 and allowed to stand in distilled water. These highly reactive ciliates were mixed with washed, heat-treated mating type 1 organisms immediately following the final high temperature period. Conjugation occurred within four hours at 35°C, the same time required for conjugation of controls (untreated mating types 1 and 2 also washed, but not mixed, 3–4 hours prior to heat-treatment of the culture of mating type 1 that was to be synchronized).

All observations of the major events during and after temperature cycling made with mating type 1, variety 1 are compatible with the theory of Scherbaum [5, 6] that blockage of division is dependent upon the reversible heat-denaturation of a single enzyme system. It is postulated here that this enzyme is concerned intimately with the movements of cell organelles involved in nuclear duplication and morphogenesis; the chromosomes and kinetosomes. Blockade by high temperature occurs *after* chromosome and kinetosome duplication, *prior* to the completion of the movement of the chromosomes to the poles of the division figure, and *before* organization of the anarchic field of kinetosomes of the anlagen into a functional mouth.

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[1] Synonymous with *T. pyriformis* WH-14.

NON-PROTEIN SULFHYDRYL COMPOUNDS IN THE DIVISION OF EGGS OF *STRONGYLOCENTROTUS PURPURATUS*[1]

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Sulfhydryl compounds have long been considered to occupy a central role in the process of cell division [5, 9, 3, 7, 10]. There are several studies of the changes occurring in non-protein SH during mitosis. Rapkine [9] has observed, by both qualitative and quantitative methods, a marked lowering of the non-protein SH content of eggs of *Paracentrotus lividus* halfway through the mitotic cycle, followed by an abrupt rise shortly before cleavage. La Grutta and Infantellina [6] have obtained a curve of completely different shape; however, their technique has been severely criticized by Bolognari [2] whose results essentially confirm those of Rapkine.

Because of the significance attached to this question, we have undertaken to study the fate of non-protein SH compounds during the first mitotic cycle of eggs of *Strongylocentrotus purpuratus*.

Experimental.—The animals were induced to shed by injection of 0.5 *M* KCl. Eggs from 4–6 females were pooled and suspended in sea water, to a concentration of about 0.3 g wet weight of eggs per 10 ml of suspension. After insemination, the eggs were continuously agitated by gentle stirring at 17–18°C. Aliquots of 10 ml were removed at intervals, and the eggs were sedimented at room temperature at low speed. The time of centrifugation (3–4 minutes) is included in the development time. The sea water was siphoned off, and the eggs were rapidly frozen as the centrifuge tube was immersed into a dry-ice isopropanol bath. This technique of stopping cell division was chosen as it was expected to result in a minimal change in the concentration of sulfhydryl constituents, either from chemical or enzymatic activity.

Samples were kept frozen at −10°C until one entire series had been collected, but no longer than three hours. They were then ground with 5–6 ml of cold dilute acid in a Ten Broeck glass homogenizer, until microscopic observation showed no unbroken cells. In three experiments, 2 per cent trichloracetic acid was used, while 3 per cent metaphosphoric acid was used in four others. The slurry was centrifuged at 8000 × *g* for 15 minutes, and the supernatant used for analysis of non-protein sulfhydryl compounds. The extraction and centrifugation were carried out at temperatures close to 0°C in order to minimise oxidation of sulfhydryl groups. It was unexpectedly found that sulfhydryl compounds (added glutathione as well as the SH compounds of the egg) disappear much more rapidly in the trichloracetic acid extract of the eggs than in the same extract neutralized to pH 6–7 (presumably by oxidation).

[1] This work was aided by a grant from the American Cancer Society and by a contract from the Office of Naval Research.

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This is in marked contrast to the behavior of aqueous solutions of cysteine and glutathione which are more resistant to oxidation at low pH.

The trichloracetic acid extracts were used for the determination of SH groups by their ability to reduce ferricyanide under mild conditions [1]. An aliquot of extract (neutralised with Na_2CO_3 water to 0.4 ml, 0.1 ml of 1 *M* tris(hydroxymethyl)aminomethane–HCl buffer, pH 6.8–7.0, and 0.5 ml of 0.0015 *M* $K_3Fe(CN)_6$ were incubated at room temperature for 10 minutes; 2.0 ml of Fe^{+++} reagent was then added (1.5 g $FeNH_4(SO_4)_2$, 1 g Duponol in 1 l of 0.05 *N* H_2SO_4 [8]), and the blue color was read at 690 mμ after 20 minutes.

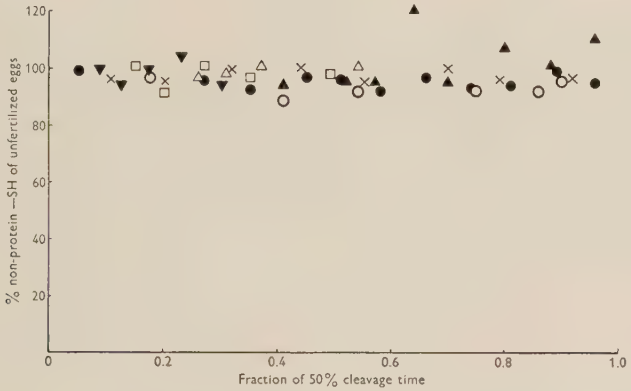

Fig. 1.—Non-protein SH as a function of the mitotic cycle. The symbols ▲, ▼, and △ refer to experiments in which the eggs were extracted with trichloracetic acid and analysed by ferricyanide oxidation. The symbols ●, ○, ×, and □ refer to experiments in which the eggs were extracted with metaphosphoric acid and analysed by the nitroprusside reaction.

The metaphosphoric acid extracts were used for analysis of sulfhydryl compounds by the nitroprusside method of Grunert and Phillips [4], with such reduction in the volume of reagents as required for use in a 3 ml Beckman cuvette, and without addition of solid NaCl to the metaphosphoric acid extract.

The results are shown in Fig. 1. Because eggs used in different experiments varied in SH content and in time schedule, the sulfhydryl concentrations are expressed as the percentage of the SH content of unfertilized eggs, and the time factor is expressed as the fraction of the time required for 50 per cent cleavage, to allow direct comparison between several series of experiments. The SH content of unfertilized eggs varied from 4–7 micromoles per gram wet weight, while the 50 per cent cleavage time varied from 92–103 minutes.

It can be seen from Fig. 1 that the non-protein SH recovered from the eggs of *Strongylocentrotus purpuratus* does not fluctuate significantly during the first mitotic cycle. This finding differs from the data of Rapkine [9] and Bolognari [2], and from the preliminary results reported some time ago from this laboratory [7]. It must be remembered, however, that some of the non-protein SH compounds may exist in the living cell as labile derivatives that are cleaved by our method of extraction and assay.

It is also possible that the gross changes observed by Rapkine and Bolognari in eggs of *Paracentrotus lividus* reflect metabolic changes peculiar to this organism, and are not necessarily linked to the processes of cell division. Alternatively, one may speculate that the non-protein sulfhydryl compounds are used for specific reactions in the mitosis of *Strongylocentrotus purpuratus* [7] but are so rapidly regenerated that no gross changes are observable. Present knowledge does not allow us to discriminate between these possiblities.

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EVIDENCE FOR THE CYTOPLASMIC FORMATION OF HEMOGLOBIN

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During our work on the biosynthesis of porphyrin and hemoglobin in rabbit reticulocytes *in vitro*, we have repeatedly observed that the particulate matter of the reticulocytes after removal of the soluble hemoglobin by hemolysis at 0–4°C, high speed centrifugation and washing with different solvents, always contained relatively high amounts of a red pigment.

It has been shown, by means of absorption microspectroscopy [8], that the pigment is a heme complex. The heme group was extractable from the particulate matter by HCl/acetone [1], and was then identified as a protoheme.

This observation was long thought to be an indication that cytoplasmic hemoglobin was formed via a nucleoprotein-intermediate. This assumption was supported by the work of Rabinovitz and Olson [7] in which evidence was presented that a ribonucleoprotein is an intermediate in the synthesis of globin by reticulocytes *in vitro*.

TABLE I. *Incorporation of methyl-^{14}C-acetate into microsome heme and cytoplasm heme of rabbit reticulocytes* in vitro.

Incubation time in minutes	c/min/mg hemin at zero thickness	
	microsome	cytoplasm
4	4.0	4.2
8	8.4	10.4
20	23.8	21.1

However, our own results during a study of the formation of the heme group in hemoglobin strongly suggested that the final formation of the heme group is a cytoplasmic process and not connected with the particulate matter of the reticulocytes. It was therefore necessary to exclude or verify this latter possibility.

If the hemoprotein held in the particulate matter actually is a ribonucleoprotein complex and a precursor to cytoplasmic hemoglobin, this should be easily proved simply by following the incorporation of ^{14}C into the particulate and cytoplasmic heme as related to time, when reticulocytes are incubated with one of the known precursors to heme-protoporphyrin labeled with ^{14}C.

If particulate heme was a precursor to cytoplasmic heme, the dilution of the incorporated ^{14}C would be negligible in the particulate heme as compared with the cytoplasmic heme. The specific activity of the particulate heme would then be expected to be of the order of that of the free protoporphyrin formed from the added ^{14}C labeled precursor.

As the specific activity of newly formed protoporphyrin in the systems used was found to be approximately 1000 times that of cytoplasmic heme protoporphyrin, one would expect to find very marked differences. Hence, trapping of small amounts of cytoplasmic hemoglobin in the particulate matter during the preparation of the cellular fractions should be of no importance for the interpretation of the results. The following experiments were performed.

Experimental.—Reticulocytes were obtained from rabbits made anemic by daily subcutaneous injections of 25 mg of phenylhydrazine hydrochloride for 5 days. The animals were killed on the 7th day by bleeding. The blood was collected over heparin, and the cells separated by centrifugation, washed twice with 0.9 per cent NaCl, and incubated in 2 volumes of 0 9 per cent NaCl/glycine (0.032 *M*).

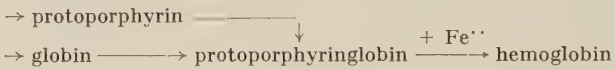
Acetate labeled in the methyl group with ^{14}C was added after temperature equilibration at 37.5°C for 5 minutes, and samples were taken after 4, 8 and 20 minutes. The samples were immediately frozen in a dry ice/acetone bath. After partial thawing, the cells were disrupted in an all glass homogenizer keeping the temperature between 0 and 4°C. The homogenates were separated into three fractions by a method slightly modified from that of Rabinovitz and Olson [7]. The coarse green-grey bottom material was discarded, while the translucent, bright red microsome rich layer and the soluble hemoglobin fraction were split into hemin and protein by treatment with 5 per cent 3 *N* HCl in acetone.

Hemin was prepared, plated and counted, as previously described [2].

Results and discussion.—As seen from Table I the specific activities are fairly identical within each pair of hemin preparations. This implies that microsomal heme cannot be a precursor to cytoplasmic hemoglobin.

Whether the microsomal hemoglobin has any biological importance at all or is just an artefact due to the preparation cannot be stated at present. Our results conclusively show that the final process in hemoglobin biosynthesis must be cytoplasmic, but do not exclude the possibility that the globin part of the hemoglobin molecule is formed via a ribonucleoprotein intermediate as suggested by Rabinovitz and Olson [7]. The evidence of Rabinovitz and Olson is very suggestive and is supported by the observation of Holloway and Ripley [6] that the incorporation of ^{14}C labeled leucine into the proteins of rabbit reticulocytes is closely related to the amount of ribonucleic acid in the cells.

We have shown previously [3, 4, 5] that the formation of hemoglobin from protoporphyrin, iron and globin most probably takes place according to the following scheme.



According to the evidence presented above the incorporation of iron into the heme group of hemoglobin takes place in the cytoplasm, while the formation of the protoporphyrin-globin complex either takes place after the release of the globin from the particulate ribonucleic acid into cytoplasm or prior to this process.

Preliminary results indicate that the latter possibility is the most probable. Further investigation of this problem is in progress.

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PROTECTIVE EFFECT OF 2-MERCAPTOETHYLGUANIDINE ON BONE MARROW CELLS X-IRRADIATED *IN VITRO*

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In studies by Doherty and coworkers [3, 4, 8], certain aminoalkylisothiuronium salts gave up to 100 per cent 30-day survival in mice exposed to lethal doses (800 r) of X radiation. These investigators made detailed studies of the reactions (in solution) of one of these compounds, S,2-aminoethylisothiuronium chloride hydrochloride (AET·Cl) and found that at pH 7–8 AET·Cl undergoes rapid intratransguanylation to form 2-mercaptoethylguanidine hydrochloride (MEG·Cl). The present report deals primarily with the protective effect of MEG·Cl on mouse bone marrow cells X-irradiated *in vitro*.

Suspensions of bone marrow cells were irradiated in the presence or absence of MEG·Cl and then injected into lethally X-irradiated mice. The 30-day survival of the irradiated mice was the basis for judging the protective ability of the compound since 90–100 per cent of lethally irradiated (900 r) mice injected with normal isologous bone marrow survive for 30 days and all irradiated mice not so treated die by the thirtieth day [6]. Bone marrow promotes recovery by repopulating the damaged hematopoietic tissue of the irradiated recipient (see review by Congdon [2]). Since repopulation requires viable cells, the test described in this paper becomes a measure of the viability of bone marrow cells exposed to X rays with or without a protective agent.

Materials and methods.—Femoral bone marrow of normal (101 × C3H) F_1 male mice was suspended in isologous plasma. Saline alone or medium 199 plus 20 per cent horse serum was sometimes substituted for plasma. The marrow cell concentration was about 20×10^6 cells per milliliter (equivalent of marrow from 2 femurs). An aqueous solution of MEG·Cl (weighed out as AET·Cl), pH 7.1–7.25, was added to the cell suspension and the preparation allowed to stand 15 minutes before X irradiation in Lusteroid tubes. Four concentrations of MEG·Cl in the irradiated preparation were tested, ranging from 3.25×10^{-4} to 1.30×10^{-2} *M*. The procedures were carried out at room temperature (25°C). Bone marrow cell suspensions without MEG·Cl were irradiated simultaneously in all *in vitro* experiments. Within 2 hours after whole-body X irradiation (900 r), each male mouse of strain (101 × C3H) F_1 was injected intravenously with a one-femur equivalent (0.5 ml/mouse) of an irradiated preparation. The 30-day survival was recorded. The amino acid, cysteine, was similarly tested for its protective action on irradiated bone marrow cells.

In other experiments, bone marrow from donor mice injected with MEG before whole-body irradiation was tested for its ability to promote recovery in irradiated recipient mice.

[1] Operated by Union Carbide Nuclear Company for the U.S. Atomic Energy Commission.

TABLE I. *The protective effect of MEG · Cl on X-irradiated mouse bone marrow cells as judged by their ability to promote recovery in mice exposed to 900 r.*

	X-ray dose to marrow cells *in vitro* (r)	Molar concn. of compound in irradiated preparation	No. of mice in group	30-day survival (%)
Bone marrow[a]				
a. Normal[b]	0	0	62	95
b. Without protective compound[b]	800	0	75	0
c. With MEG added before X ray	800	3.25×10^{-3} to 1.30×10^{-2}[c]	40	95
d. With MEG added before X ray	800	3.25×10^{-4}	10	0
e. With MEG added before X ray	1500	1.30×10^{-2}	7	43
f. With MEG added before X ray	1700	1.30×10^{-2}	7	0
g. With MEG added before X ray (in medium 199 + horse serum)	800	1.30×10^{-2}	13	45
h. With MEG added before X ray (in saline)	800	1.30×10^{-2}	16	13
i. With MEG added after X ray	800	1.30×10^{-2}	12	0
j. Taken, on day 0, from irrad. mice protected with MEG	0	—	10	100
k. Taken, on day 3, from irrad. mice protected with MEG	0	—	8	75
l. With cysteine added before X ray	800	1.30×10^{-2}	12	100
Irradiated (900 r) uninjected control mice[b]	—	—	90	0

[a] Suspension medium was isologous plasma unless otherwise noted.
[b] Cumulative for all experiments.
[c] 3.25×10^{-3}, 6.25×10^{-3}, and 1.30×10^{-2} *M* concentrations were used in six different experiments.

Uninjected irradiated mice were included in each experiment. Radiation factors for both *in vitro* and *in vivo* exposures were 250 kv, 15 ma, hvl of 0.5 mm Cu, and a dose rate in air of approximately 160 r/minute.

Results.—(*Table I.*) Ninety-five per cent of the irradiated mice injected with normal isologous bone marrow survived 30 days or more (*a*). Bone marrow exposed to 800 r *in vitro* lost ist capacity to promote recovery in the irradiated mice (*b*). MEG added 15 minutes before exposure, however, protected the cells, as evidenced by the 95 per cent 30-day survival (*c*). MEG was effective at concentrations of 3.25×10^{-3}, 6.25×10^{-3}, and 1.30×10^{-2} *M* but was ineffective at 3.25×10^{-4} *M* (*d*). Some protection of the marrow cells exposed to 1500 r in the presence of MEG was observed (*e*), but protection was not apparent at 1700 r (*f*). If medium 199 plus horse serum or a medium of 0.9 per cent saline was substituted for isologous plasma, the effectiveness of MEG was greatly reduced (*g* and *h*). When MEG was added to the bone marrow

suspension within 10 seconds after irradiation and the preparation allowed to stand 15 minutes before injection, protection was not afforded the marrow cells (*i*). Bone marrow from mice injected with MEG intraperitoneally before whole-body irradiation (800 r) retained its ability to promote recovery whether the marrow was injected immediately (*j*) or 3 days (*k*) after irradiation. In two experiments, cysteine also afforded protection to irradiated bone marrow cells (*l*).

Discussion.—The results of these experiments show that the effectiveness of mouse bone marrow as a recovery agent is destroyed *in vitro* by 800 r of X rays, a dose that is an LD_{100} (30 days) for male $(101 \times C3H)F_1$ mice. Moreover, the data indicate that MEG can protect bone marrow at the cellular level. When MEG is used, therefore, to protect animals from the acute lethal effects of irradiation, it is likely that the protective action is accomplished directly on cells of hematopoietic tissue. This hypothesis precludes an indirect mechanism whereby the action of MEG on bone marrow is mediated through other tissues.

MEG afforded some protection to marrow cells exposed to 1500 r. Apparently, the compound has a dose-reducing capacity, as Doherty *et al.* [4] observed when they used MEG to protect against supralethal doses of radiation to the whole body. They also found that an injection of MEG into mice after irradiation gave no protection (personal communication). Similarly, MEG added to the bone marrow suspension *after* irradiation did not protect the cells, according to the test system used in the present experiments. Since MEG is not protective if introduced *in vivo* or *in vitro* after irradiation, the MEG contained in the bone marrow suspensions probably had no protective effect on the hematopoietic tissues of the irradiated mice.

Results of these experiments show that the *in vitro* protective effect of MEG is greater in plasma than in a synthetic medium or in physiological saline. Plasma may allow more rapid incorporation of MEG by the cells or it may contain a specific substance (or substances) necessary for the protective action, or both. Whatever the conditions may be, they apparently are not met so well by the synthetic medium or the saline.

Patt *et al.* [7] showed that cysteine confers protection on thymocytes irradiated *in vitro* over a wide range of X-ray doses. They used an eosin stainability technique as a criterion for the degree of protection. Using a similar procedure, van Bekkum and de Groot [1] reported that other biological amines; e.g., cysteamine, also protects thymocytes irradiated *in vitro*.

Cytologically, there is a wide spectrum of cell types in bone marrow, a situation not well suited for studies involving quantitative relations among dose of radiation, number of marrow cells, and amount of protective compound. When bone marrow is used as a recovery agent, however, it is likely that only those cells capable of dividing are important in repopulating hematopoietic tissue of the irradiated host. Therefore, protection of these cells (blast cells?) *in vitro* by MEG was probably responsible for maintaining the recovery action of irradiated bone marrow. It also seems likely that the MEG, administered to mice before irradiation, protects these types of cells and thereby allows the animals to recover from the acute lethal effects of irradiation. This hypothesis is supported by the observation that depression in the number of nucleated bone marrow cells of a femur is less in X-irradiated mice protected with MEG than in unprotected irradiated controls [9]. Protection may also be afforded, however, to other types of blood cells as well as cells of other tissues.

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DPNH-OXIDASE ACTIVITY IN FRAGMENTS OF RAT LIVER MITOCHONDRIA[1]

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It has been possible to obtain particles with DPNH-oxidase activity by grinding rat liver mitochondria with finely powdered Al_2O_3, followed by differential centrifugation of the resulting suspension to secure the fraction which sediments between 25,000 and 100,000 $\times g$ (20 minutes centrifugation in both cases). Al_2O_3 was used for the grinding, since Smith and M. Baltscheffsky [4] had in this way obtained extracts capable of respiration and photosynthetic phosphorylation from *Rhodospirillum rubrum*. 0.03 *M* phosphate buffer was used for the operations above, since Bronk and Kielley [1] had obtained particles capable of respiration and oxidative phosphorylation by supersonic treatment of mitochondria, employing this medium.

The DPNH-oxidase activity has been measured both with the Warburg method and by following the rate of disappearance of the absorption band of DPNH at 340 mμ. Addition of cytochrome *c* (10^{-5} *M*) was necessary for maximum activity, which

[1] Abbreviations: DPNH, reduced diphosphopyridine nucleotide; TPNH, reduced triphosphopyridine nucleotide; P, erthophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; *M*, moles per liter.

[2] Supported by a grant from "Ella och Georg Ehrnrooths stiftelse".

varied between 0.2 and 0.4 μmoles of DPNH oxidized per minute per mg protein at 20°C, for three different preparations. With no cytochrome *c* added the activity was between 10 and 40 per cent of the maximum. The yield of DPNH-oxidase was about 0.6 mg of protein per 10 g of liver.

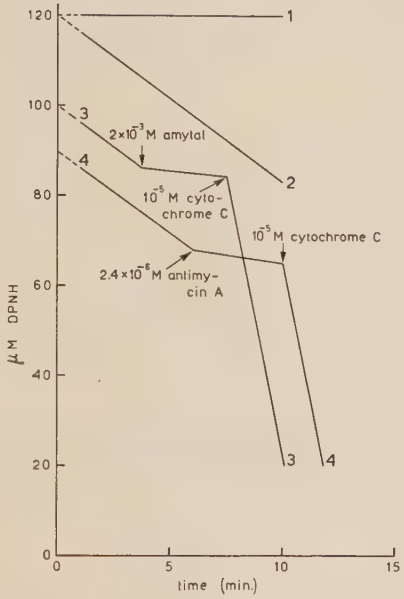

Fig. 1.—Oxidation of DPNH, measured in a Beckman DK 2 spectrophotometer supplied with a time drive attachment. *Curve 1*, 2.9 ml 0.03 *M* phosphate buffer pH 7.0 + 0.1 ml 0.005 *M* DPNH. *Curves 2*, *3* and *4*, 2.5 ml 0.03 *M* phosphate buffer + 0.1 ml 0.005 *M* DPNH + 0.4 ml DPNH-oxidase preparation. Additions are shown in the figure, where final molarities are given. The starting points for the curves have been arbitrarily chosen.

Amytal (2×10^{-3} *M*) and antimycin A (2.4×10^{-6} *M*) inhibited the oxidation of DPNH when no cytochrome *c* was added, but did not inhibit the respiration due to addition of cytochrome *c*. These measurements were made in 0.028–0.030 *M* phosphate buffer pH 7.0. The inhibition caused by amytal and antimycin A is shown in Fig. 1, which also shows that a rapid oxidation of DPNH was initiated by the addition of cytochrome *c* in the presence of these inhibitors.

The succinoxidase activity of the fragments was low (0–10 per cent, average 6 per cent, according to four experiments with different preparations) compared to the DPNH-oxidase activity. The selectivity of the alumina method for obtaining DPNH-oxidase activity constitutes a drastic difference from the ultrasonic method of Kielley and Bronk [2], which gives particles with roughly equal DPNH-oxidase and succinoxidase activities.

The rate of oxidation of TPNH, whether or not cytochrome *c* was added, was less than half the rate of oxidation of DPNH in the absence of added cytochrome *c*.

The rate of oxidation of DPNH was not stimulated by ADP, ADP + Mg^{++}, or ATP + glucose + hexokinase + Mg^{++}. It was inhibited more than 95 per cent by KCN (10^{-3} *M*).

The oxidation of DPNH was accompanied by phosphorylation, as measured with the method described by Lindberg and Ernster [3]. Table I shows that phosphorylation was obtained both in the presence and in the absence of added cytochrome *c*.

TABLE I. *Respiration and phosphorylation by DPNH-oxidase preparation. Substrates: DPNH and succinate.*

Each Warburg vessel contained 10 μmoles orthophosphate, 0.4 μmoles ATP, 10 μmoles Mg^{++}, 100 μmoles glucose and hexokinase in excess. Additions (where indicated) 5 μmoles DPNH; 0.02 μmoles cytochrome *c*, 30 μmoles succinate. Final volume 2.0 ml. Temperature 30°C. (The values for succinate are included only to show the order of magnitude.)

Additions	Respiration μatoms O	Phosphorylation μmoles P
DPNH, cytochrome *c*	2.4	0.26
DPNH	0.8	0.20
Succinate, cytochrome *c*	0.2	0.09
Antimycin A	0	0.05[a]

[a] This is the blank value which we generally obtain with the method used.

From the values obtained, it can be deduced that it is the amytal- and antimycin A-sensitive part of the respiration which is connected with the phosphorylation. This is in agreement with current concepts.

A more detailed report will be published.

Many thanks are due to Professor Olov Lindberg for his support and active interest in this work and to Miss Molly Fudge for excellent assistance.

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